WNT/β-CATENIN SIGNALING REGULATES MULTIPLE ASPECTS OF LATERAL LINE MORPHOGENESIS

by

Andrew John Aman

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Andrew John Aman**

has been approved by the following supervisory committee members:

Tatjana Piotrowski	, Chair	**8/23/2010** Date Approved
Chi-Bin Chien	, Member	**8/23/2010** Date Approved
Gabrielle Kardon	, Member	**8/23/2010** Date Approved
David Jones	, Member	**8/23/2010** Date Approved
Rich Dorsky	, Member	**8/23/2010** Date Approved

and by **Monica Vetter**, Chair of the Department of **Neurobiology and Anatomy**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

During development, groups of cells in the embryo must organize into precise morphologies required for the function of tissues and organs to enable the survival of organisms. This morphogenesis requires the coordinated regulation of basic cellular behaviors such as migration, cell shape changes and cell proliferation. Cell-cell signaling is required to orchestrate basic cellular behaviors in space and in time ensuring that these behaviors contribute to morphogenesis. Misregulated morphogenic signaling is associated with human disease. Understanding the cell-cell signaling interactions that regulate morphogenic cell behavior is a fundamental biological question.

Zebrafish primary posterior lateral line (lateral line) development is an excellent basic model of morphogenesis. The lateral line emerges from a migrating primordium that travels as a cohesive, multicellular collective from the head to the tip of the tail. Prosensory organs are formed within in the migrating primordium and deposit from its trailing edge to generate a single row of mechanosensory organs distributed along the A-P axis of the trunk.

Migration of the primordium depends on the interaction of the guidance molecule *cxcl12a*, expressed along the presumptive migratory path, and the receptors *cxcr4b* and *cxcr7b*. *cxcr4b* is expressed broadly expressed in the migrating primordium and *cxcr7b* is expressed exclusively in trailing and

deposited cells. Both receptors are necessary for directed migration of primordium cells.

Prosensory organ formation is involves the apical constriction of cells to generate cohesive, rosette shaped clusters. Rosette morphogenesis does not occur in cells occupying the leading region of the primordium, which serves as a progenitor pool for further prosensory organ formation.

Data presented in this dissertation show that Wnt/β-catenin signaling is a key coordinator of cellular behaviors that underlies lateral line morphogenesis. First, Wnt/β-catenin signaling regulates the expression of chemokine receptors that underlie directional migration. Second, Wnt/β-catenin signaling restricts Fgf dependent proneuromast formation from the leading zone of the primordium maintaining this region as a progenitor zone and ensuring complete lateral line morphogenesis. Finally, Wnt/β-catenin signaling regulates proliferation of primordium cells and proliferation, in turn, influences the periodicity of proneuromast formation. Therefore, Wnt/β-catenin signaling represents a key coordinator of morphogenetic cell behaviors in during lateral line development.

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CHAPTER 1

INTRODUCTION

Overview

During embryonic development, groups of cells organize themselves into a myriad of forms. These forms can be quite simple, such as a tubular sea sponge, or fantastically complex, such as a human being. It is known that genetic instructions execute complex programs of cell behaviors that ultimately generate the final morphology of an organism. How groups of cells organize themselves into specific, genetically encoded morphologies during development is a fundamental problem in biology.

Morphogenesis involves the precise coordination of individual cell behaviors to generate tissues, organs and organisms with specific three-dimensional geometries. Cells can exhibit a variety of dynamic behaviors including changing size and shape, migrating, proliferating, excreting extracellular matrix of various compositions and adhering to one another or to extracellular matrix (Lodish et al., 2001). At one level, morphogenesis can be understood as the end result of the combined action of such dynamic behaviors. For example, cell shape change in the form of apical constriction of epithelial cells can drive formation of cups or tubes (Sawyer et al., 2010).

Although understanding morphogenesis at this mechanical level is important, to fully understand morphogenesis requires elucidating the regulatory mechanisms that coordinate multiple cell behaviors in the embryo. Cells must communicate with each other to ensure that they execute the required behaviors at the necessary places and times to generate the functioning morphology. This communication is achieved in the embryo by various cell-cell signaling pathways.

The most prevalent and best characterized signaling pathways involve the secretion of diffusible signaling molecules that lead to changes in target cell behavior, although other strategies exist (Lecaudey and Gilmour, 2006). Cell-cell signaling can influence cell behavior in a number of ways, including regulating the expression of genes or directly affecting the function or organization of cytoplasmic proteins in the target cell (Miller and McCrea, 2010). This dissertation focuses on the problem of how cell-cell signaling coordinates cell behavior across a group of cells to regulate morphogenesis.

To investigate this problem I use development of the zebrafish primary posterior lateral line (lateral line) as a basic model. The lateral line has exceptional advantages for this study. First, cell behaviors that contribute to lateral line morphogenesis can be observed directly in the living embryo. Second, development of the lateral line is largely autonomous and can be manipulated without detriment to overall embryonic development. Third, the lateral line has a relatively simple morphology. These characteristics facilitate the elucidation of mechanistic connections between cell signaling, cell behavior and final morphology.

To exploit these advantages, a candidate screen for cell signaling pathways involved in lateral line morphogenesis was undertaken. This revealed the crucial importance of Wnt/ β-catenin signaling in lateral line morphogenesis. Functional analysis of this pathways fduring lateral line development lead to the discovery of a Wnt/ β-catenin mediated feedback system that coordinates several key morphogenic cell behaviors.

Background

Wnt/β-catenin signaling

The Wnt/β-catenin pathway has a huge number of different functions during development including the regulation of proliferation, differentiation and migration in a large number of contexts. Defects in Wnt/β-catenin signaling components underlie numerous human diseases (Clevers, 2006; Logan and Nusse, 2004). Wnt/ β-catenin signaling is conserved throughout animal evolution and is well characterized biochemically providing a host of tools with which to manipulate the pathway in model systems (MacDonald et al., 2009). In this section, I will briefly introduce pertinent details of the Wnt/β-catenin signaling pathway.

Wnt proteins are secreted signaling molecules that bind to Frz and LRP receptors in target cells (Schweizer and Varmus, 2003). This binding triggers a cascade of interactions that results in stabilization of cytoplasmic β-catenin by disassembling the β-catenin destruction complex (Rubinfeld et al., 1996). This allows β-catenin to accumulate in the cytoplasm, facilitating its transport into the nucleus where it binds to transcriptional regulators of the TCF/LEF family. β-catenin binding converts TCF/LEF proteins from transcriptional repressors to activators inducing the expression of target genes (Figure 1.1; Clevers, 2006; Logan and Nusse, 2004).

In this dissertation, I use several tools to manipulate the state of Wnt/β-catenin signaling. To constitutively activate the pathway I use a zebrafish *apc* mutant (Hurlstone et al., 2003). Apc is a crucial component of the β-catenin

destruction complex, and thus, in the absence of functional Apc the signaling pathway can be constitutively activated in target cells (Figure 1.1; Bienz, 2002; Rubinfeld et al., 1996).

To reduce Wnt/β-catenin activity I use a heatshock inducible *dkk1* transgenic that is capable of inhibiting the binding of Wnt to LRP receptors (Stoick-Cooper et al., 2007). A second tool used to downregulate Wnt/β-catenin signaling involves heatshock expression of a dominant repressor form of *tcf* (Lewis et al., 2004). This molecule can bind DNA and repress target gene expression but cannot bind β-catenin and, therefore, cannot activate gene expression. In Chapter 2 these reagents are shown to be effective in manipulating Wnt/β-catenin signaling during lateral line development.

The zebrafish posterior lateral line

The lateral line is a mechanosensory system found in aquatic vertebrates that allows detection of water movements around the animal. The relative simplicity and accessibility of the developing zebrafish primary posterior lateral makes it a powerful model to resolve molecular mechanisms regulating morphogenesis (Dambly-Chaudiere et al., 2007). In this section, I will introduce lateral line development paying special attention to the basic cell behaviors that drive morphogenesis.

The lateral line is composed of rosette shaped mechanosensory organs called neuromasts distributed across the surface of the animal (Stone, 1922; Metcalfe et al., 1985). Each neuromast contains three principal cell types: Mechanosensory hair cells that are structurally and functionally similar to the hair

cells that underlie hearing in terrestrial vertebrates occupy the center of the neuromast. Support cells and mantle cells lie at the periphery surrounding the hair cells (Gompel et al., 2001). Hair cells detect motion of water directly, via a mechanism involving the directional deflection of a ciliary bundle extended from their apical aspect (Gillespie and Müller, 2009). In zebrafish, all neuromasts are situated in the dermis such that the ciliary bundle extends directly into the environment where it can be deflected by water motion. In other species, neuromasts can reside in bony canals that are open to the environment (Webb, 1989).

The lateral line can be subdivided into two main branches: the anterior and posterior lateral line. Neuromasts of the anterior lateral line cover the head of the animal and neuromasts of the posterior lateral line are distributed on the trunk (Gompel et al., 2001; Webb, 1989). The posterior lateral line can be further subdivided into several groups of neuromasts defined by embryonic origin (Sapede et al., 2002; Ghysen and Dambly-Chaudiere, 2004). The primary posterior lateral line is the first group of neuromasts to develop along the horizontal myospetum (Gompel et al., 2001). During postembryonic development, repeated waves of neuromast formation occur on the trunk so that neuromasts become evenly distributed across the surface of the mature animal (Grant et al., 2005; Nuñez et al., 2009; Sapede et al., 2002). The work in this dissertation pertains to the primary posterior lateral line which will be referred to simply as 'the lateral line.'

The lateral line develops from a placode that forms immediately posterior to the otic placode (Gompel et al., 2001; Stone, 1922). Shortly after delamination, the posterior lateral line placode begins to proliferate and elaborate extensions becoming the lateral line primordium (Sarrazin et al. 2010). Once formed, the primordium migrates posteriorly along the horizontal myoseptum to the tip of the tail (Figure 1.2A-D). During migration, cells within the trailing region of the primordium organize into rosette shaped proneuromasts that are periodically deposited from the trailing edge of the primordium (Figure 1.2E,F; Gompel et al., 2001). In addition, the primordium deposits a single continuous line of cells, called interneuromast cells, between deposited proneuromasts (Figure 1.2C; Grant et al., 2005). These processes generate a simple morphology at the end of embryonic development composed of a single row of proneuromasts distributed along the horizontal myospetum (Figure 1.2D; Gompel et al., 2001; Metcalfe et al., 1985).

Important cell behaviors for the morphogenesis of the lateral line include cell migration, cell shape changes, dynamic cell-cell adhesion and cell proliferation. I will next summarize what is known about these processes in lateral line development.

The primordium migrates as a cohesive, multicellular collective composed of approximately 100 cells (Gompel et al., 2001). During migration, all cells in the primordium extend processes along the migratory path and presumably contribute to traction forces. Protrusions are easily observed In cells at the leading edge of the primordium. Trailing cells require mosaic labeling techniques

to observe their extensions (Haas and Gilmour, 2006). Migration of primordium cells is coordinated so that all cells are moving in the same direction at the same speed. As a result, cells within the primordium do not frequently exchange positions, as revealed by time-lapse microscopy (Haas and Gilmour, 2006).

Chemokine signaling is the primary guidance system responsible for directing the primordium along its migratory path. The chemokine receptor *cxcr4b* is broadly expressed in the migrating primordium but is not expressed in deposited proneuromasts or interneuromast cells and the guidance ligand *cxcl12a* (*sdf1a*) is expressed along the migratory path. Loss of function of either gene causes a failure of migration (David et al., 2002) and misexpressing *cxcl12b* causes the primordium to migrate to ectopic locations (Li et al., 2004). Interestingly, in the absence of *cxcl12a* or *cxcr4b* individual primordium cells will attempt to migrate in random directions leading to cell tumbling within the primordium and a rounded primordium morphology. Therefore, chemokine signaling is not necessary for motility of primordium cells but only for their guidance (Haas and Gilmour, 2006).

A second Cxcl12a binding receptor, *cxcr7b*, is expressed in the trailing portion of the tissue. This receptor is also necessary for directional migration as loss of function causes failure of migration and cell tumbling. Distinct from loss of *cxcl12a* or *cxcr4b*, however, loss of *cxcr7b* does not cause cell tumbling uniformly throughout the primordium. Rather, cells at the leading edge continue attempting directional migration, while the remainder of cells tumble leading to an

elongated primordium morphology rather than a rounded morphology (Dambly-Chaudiere et al., 2007; Valentin et al., 2007).

It is thought that Cxcr7b in the trailing portion of the primordium acts as a sink for Cxcl12a, analogous to the role of this molecule in guiding migrating primordial germ cells (Boldajipour et al., 2008; Dambly-Chaudiere et al., 2007). By binding Cxcl12a and removing it from extracellular space it is thought that Cxcr7b helps generate a gradient of Cxcl12a across the migrating primordium, thereby allowing guided collective migration.

Chapter 1 provides evidence for a signaling feedback network involving Wnt/β-catenin and Fgf signaling that maintains the polarized expression of chemokine receptors. Chapter 2 discusses in more detail how chemokine receptor asymmetry is thought to underlie collective migration and highlights potential limitations of the Cxcl12a sink hypothesis for the function of Cxcr7b. Chapter 3 compares and contrasts the regulation of primordium migration with several other examples of morphogenic cell migration.

During its journey, the primordium will sequentially deposit five or six clusters of cells fated to give rise to mechanosensory neuromasts (Figure 1.1D). These clusters form as rosette shaped proneuromasts within the migrating primordium so that a primordium typically houses two or three rosettes as it migrates (Figure 1.2E.F; Gompel et al., 2001; Haas and Gilmour, 2006). Proneuromasts are formed toward the leading edge and are deposited from the trailing edge (Gompel et al., 2001; Metcalfe et al., 1985). Therefore, the rate of

primordium migration, proneuromast formation and proneuromast deposition will all influence the final spacing of proneuromasts.

Proneuromast formation requires the action of Fgf signaling, which likely regulates epithelial apical-basal polarity regulators such as *lgl2* and *prkc*. Activation of apical-basal polarity machinery causes cells in a presumptive proneuromast to constrict their apical aspects into a central point, producing the rosette morphology of the proneuromast (Hava et al., 2009; Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Additionally, Fgf signaling, in coordination with Delta-Notch signaling, functions to specify hair cell precursors in the primordium (Itoh and Chitnis, 2001; Lecaudey et al., 2008; Nechiporuk and Raible, 2008). It has been proposed that apical constriction during proneuromast rosette formation leads to the accumulation of adhesion molecules between cells within the proneuromast. This may contribute to the cohesiveness of the proneuromast as it is deposited (Hava et al., 2009).

Therefore, in addition to migration, cell shape change in the form of apical constriction of proneuromast cells within the primordium is another critical cell behavior underlying lateral line morphogenesis. Data in Chapter 2 demonstrate that Wnt/β-catenin signaling restricts proneuromast formation to the trailing region of the primordium by regulating the distribution of Fgf signaling.

The migrating primordium consists of approximately 100 cells and deposits 4-6 proneuromasts each comprised of approximately 20 cells (Gompel et al., 2001). Despite this, the primordium does not rapidly dwindle during migration and deposition (asterisks in Figure 1.1C-E; Gompel et al., 2001).

Therefore, the primordium must precisely coordinate cell proliferation with migration and proneuromast deposition. If this coordination were to fail it is possible that the primordium would not be able to maintain enough cells to generate the normal morphology of the lateral line.

Patterns of proliferation in the primordium have been studied by BrdU incorporation experiments. These studies show that there is a higher rate of BrdU incorporation in the leading portion of the primordium, whereas the trailing portion of the tissue is relatively quiescent (Laguerre et al., 2005). The regulation of proliferation within the primordium and the morphogenic effect of proliferation on the lateral line are not known. By labeling small groups of leading edge cells immediately prior to primordium migration and following the fate of the labeled cells (Nechiporuk and Raible, 2008) found that label was eventually detected throughout the primordium and in deposited tissue. This demonstrates that the leading region of the primordium harbors a pool of proliferative progenitor cells. Chapter 4 provides evidence that proliferation is regulated by Wnt/ β-catenin in the primordium and that the rate of proliferation is a crucial determinant of the rate of proneuromast deposition.

In summary, this dissertation provides evidence that Wnt/β-catenin signaling regulates three crucial cell behaviors during lateral line morphogenesis: directed migration, cell shape changes and cell proliferation. This ensures that these cell behaviors are executed in a coordinated way so as to generate the correct morphology of the lateral line.

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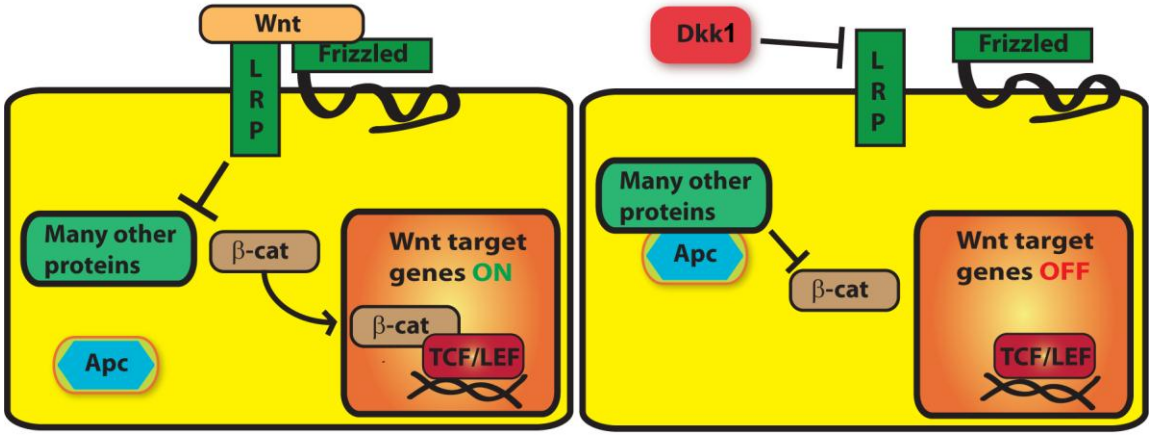

Figure 1.1. Simplified schematic of Wnt/β-catenin signaling.
(Left) Wnt binding to LRP and Frizzled co-receptors leads to distruption of the β-catenin destruction complex formed by interactions between Apc and many other proteins. This allows β-catenin to accumulate in the cytoplasm (yellow) facilitating its transport into the nucleus (orange) where it interacts with TCF/LEF family transrition factors and activates expression of target genes. (right) In the absence of Wnt ligand, the β-catenin destruction complex prevents cytoplasmic accumulation of β-catenin. Dkk is a diffusable inhibitor of Wnt/β-catenin signaling that acts by repressing Wnt interaction with LRP receptors.

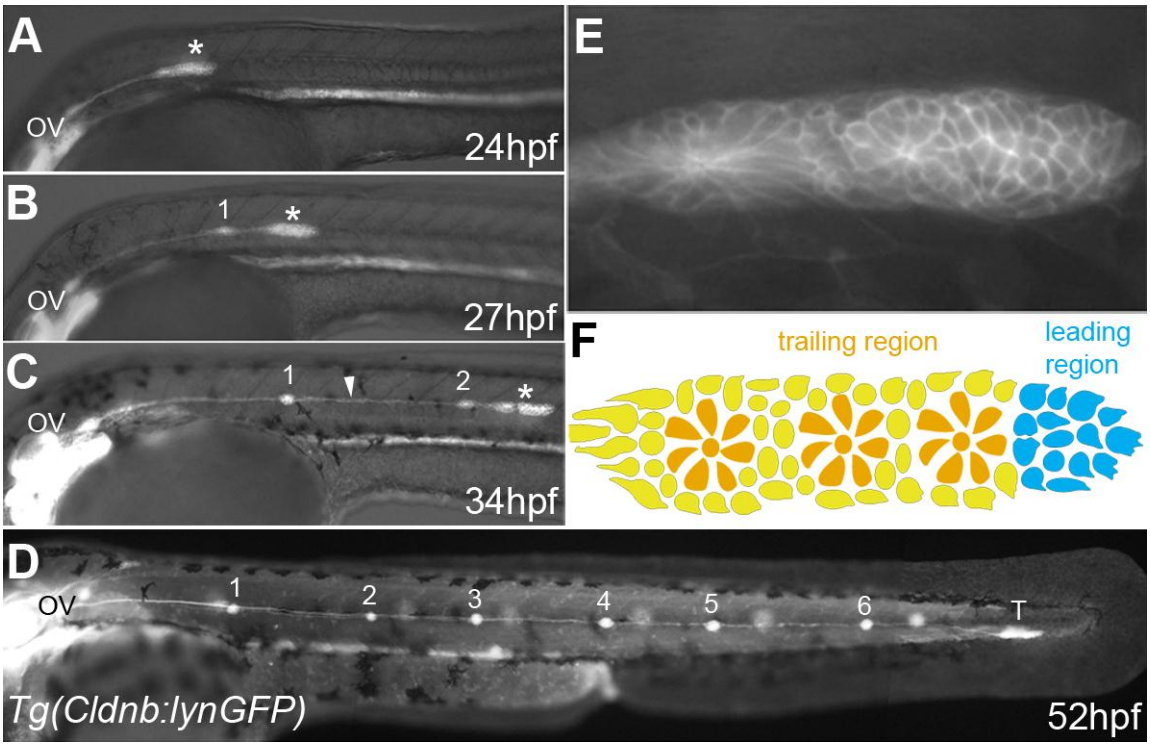

Fig. 1.2. Embryology of the lateral line

(A-E) Lateral line embryology visualized using *Tg(cldnb:lynGFP)* embryos. (A) At 24hpf the primordium (asterisk) has fully migrated onto the somites from its origin near the otic vesicle (OV). (B) The primordium has recently deposited the first proneuromast. (C) The second proneuromast is deposited by 34hpf, interneuromasts are labeled with a white arrowhead. (D) the post embryonic lateral line is comprised of 4-6 proneuromast in a row along the horizontal myoseptum. (E) Higher magnification of the primordium in panel A. Note the rosette shaped proneuromasts forming in the trailing region. (F) Schematic of proneuromast formation within the primordium. The blue leading region is remains free of proneuromasts throughout migration and represents a progenitor pool. Rosette shaped proneuromasts form and are deposited from the yellow/orange trailing region.

CHAPTER 2

WNT/β-CATENIN AND FGF SIGNALING CONTROL COLLECTIVE CELL MIGRATION BY RESTRICTING CHEMOKINE RECEPTOR EXPRESSION

The following chapter is reprinted with permisson from:

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Summary

Collective cell migration is a hallmark of embryonic morphogenesis and cancer metastases. However, the molecular mechanisms regulating coordinated cell migration remain poorly understood. A genetic dissection of this problem is afforded by the migrating lateral line primordium of the zebrafish. We report that interactions between Wnt/β-catenin and Fgf signaling maintain primordium polarity by differential regulation of gene expression in the leading versus the trailing zone. Wnt/β-catenin signaling in leader cells informs coordinated migration via differential regulation of the two chemokine receptors *cxcr4b* and *cxcr7b.* These findings uncover a novel molecular mechanism whereby a migrating tissue maintains stable, polarized gene expression domains despite periodic loss of whole groups of cells. Our findings also bear significance for cancer biology. Although the Fgf, Wnt/β-catenin and chemokine signaling pathways are well known to be involved in cancer progression, these studies provide the first in vivo evidence that these pathways are functionally linked.

Introduction

Cell migration is a fundamental, tightly coordinated process during the embryonic and adult life of animals. Organogenesis, wound healing and immune responses, for example, are characterized by a robust and exquisite orchestration of directed movements of cells toward specific locations. In some cases, cells migrate as individuals, *e.g.*, neural crest cells and cells comprising our immune system (De Calisto et al., 2005; Redd et al., 2006). In other cases, cells migrate as groups adhering to each other via cell-cell adhesion molecules

(Friedl, 2004; Rorth, 2007). Movement of groups of cells occurs, for example, during gastrulation, blood vessel formation, wound healing and Drosophila border cell migration (Martin and Parkhurst, 2004; Montell, 2006; Rorth, 2007; Schmidt et al., 2007; Solnica-Krezel, 2006). While much has been learned about how individual cells migrate from *in vitro* studies, the mechanisms integrating migration and morphogenesis of groups of cells *in vivo* are among the least understood processes in developmental biology. Important questions awaiting satisfactory mechanistic explanations include how cluster polarity is maintained and how tip cells communicate with cells in the back to ensure coordinated, directed migration. Elucidating the mechanisms regulating collective cell migration is not only crucial for our understanding of morphogenesis, but is also highly relevant to cancer biology as several human cancers, including breast and prostate cancer, invade tissues as groups of cells (Friedl et al., 2004; Hegerfeldt et al., 2002).

The relative simplicity and experimental accessibility of the zebrafish lateral line provide a robust model for elucidating mechanisms that regulate collective cell migration (Ghysen and Dambly-Chaudiere, 2004). The lateral line is a sensory system found in aquatic vertebrates that detects water movements. It consists of mechanosensory organs called neuromasts arranged in rows along the flanks of the animal (Metcalfe et al., 1985; Northcutt et al., 1995; Platt, 1896; Schulze, 1861; Stone, 1922). Mature neuromasts are composed of mechanosensory hair cells in the center, and supporting cells and mantle cells at the periphery. These sensory organs arise from a neurogenic placode that forms

posterior to the otic placode and delaminates to become the migrating primary lateral line primordium. During migration, neuromast precursors (proneuromasts) are sequentially deposited from the trailing zone of the primordium approximately every 3-5 somites (Figure 2.1A; (Gompel et al., 2001; Metcalfe et al., 1985). It has been suggested that the directionality of this collective cell migration is not controlled by a gradient of an extrinsic guidance molecule but rather by the polarized expression of the two chemokine receptors *cxcr7b* and *cxcr4b* within the primordium (Dambly-Chaudiere et al., 2007; Haas and Gilmour, 2006; Valentin et al., 2007). To date, however, the molecular mechanisms that establish and maintain this expression asymmetry during migration are not understood. Primordium polarity also underlies proneuromast formation and deposition from the trailing zone of the primordium. Cells in the leading third of the primordium are unpatterned, whereas trailing cells are organized into rosette-shaped proneuromasts and express proneural and neurogenic genes preceding hair cell differentiation (Itoh and Chitnis, 2001; Lecaudey et al., 2008; Nechiporuk and Raible, 2008).

Our studies have identified important cell-cell signaling events that occur between cells in the leading and trailing zones of a migrating cell cluster that function to maintain its polarity as it migrates. The network is based on localized activation of the Wnt/β-catenin pathway in the leading zone of the primordium. Subsequent interactions between the Wnt/β-catenin and Fgf signaling pathways serve to restrict activation of these pathways into mutually exclusive domains. Restricted activation of Wnt/β-catenin signaling controls the localized expression

of *cxcr4b* and *cxcr7b* and coordinates cell migration with sensory organ deposition by restricting Fgf signaling to trailing cells.

Results

Misregulation of the Wnt/β-catenin signaling pathway causes cell migration defects

To investigate a possible role of Wnt/β-catenin signaling in lateral line development, we analyzed a recessive zebrafish mutation in *apc* (*adenomatous polyposis coli;* apc^{mcr} Hurlstone et al., 2003; Peifer and Polakis, 2000). APC is a scaffolding protein with several protein binding domains (Nathke, 2005). It is best known for regulating the Wnt/β-catenin signaling pathway in which it is a necessary component of the complex that targets β-Catenin for destruction in the absence of active Wnt signaling (Bienz, 2002; Rubinfeld et al., 1996). About 85% of human colon cancer patients possess mutations in *APC* that lead to constitutive activation of the Wnt/β-catenin signaling pathway in affected cells (Kinzler and Vogelstein, 1996; Reya and Clevers, 2005). In addition to its role in regulating the Wnt/β-catenin pathway, *in vitro* studies of migrating cells have shown that association of the C-terminus of APC with microtubules is necessary for normal migration (Kroboth et al., 2007). Similarly to the majority of mutations in human APC, the zebrafish apc^{mcr} mutation truncates the protein deleting the central and C-terminal domains necessary for regulating Wnt/β-catenin signaling and normal cytoskeletal association, respectively (Figure 2.1O; Hurlstone et al., 2003).

We characterized lateral line primordium development in wildtype (wt) and *apc*^{mcr} mutant embryos using static and dynamic assays. *In situ* hybridization with *eya1*, a lateral line marker, at 36hpf demonstrates that the mutant primordium does not reach the tail tip (Figure 2.1A,B). Wt primordia travel at a constant speed periodically depositing proneuromasts, whereas *apc*^{mcr} mutant primordia stall at around 27-29 hpf after leaving behind a disorganized band of cells (Figs. 2.1A-H). Individual cells within the *apc*^{mcr} mutant primordium are motile but fail to undergo directed coordinated cell migration. Tip cells in the leading zone of wt primordia respond to guidance cues and are important for directional migration toward the tail tip (Figure 2.1I; Haas and Gilmour, 2006). In stalling *apc*^{mcr} mutant primordia, tip cells attempt to migrate posteriorly and elongate in the process but are held back by the remaining primordia cells which tumble randomly (Figure 2.1J; data not shown). These findings suggest that *apc*^{mcr} mutant primordia fail to migrate due to a loss of directed cell migration in trailing cells in the presence of normal tip cells. Irrespectively, proneuromasts form within the mutant primordium and deposited cells differentiate to form all the cell types normally present in neuromasts, such as support and hair cells, as revealed by *klf4* and *brn3c* expression, respectively (Figure 2.1K-N).

To determine which Apc functions are necessary for normal lateral line morphogenesis, we injected mRNA coding for the central zone of the human *APC* gene (APC-GFP; Figure 2.1O) into one cell stage embryos from a cross of *apc*^{mcr} heterozygotes (Miller and Moon, 1997). APC-GFP encodes domains necessary for regulation of β-Catenin but lacks domains needed for microtubule

association (Barth et al., 2002; Zumbrunn et al., 2001). Among 115 injected embryos, only 2 had the characteristic *apc*^{mcr} lateral line phenotype as assayed by incorporation of DASPEI, a vital dye that labels mechanosensory hair cells and *eya1 in situ* hybridization. All other injected *apc*^{mcr} homozygotes were indistinguishable from wt embryos (Figure 2.1P,Q). In contrast, uninjected embryos from the same cross contained the expected frequency of mutants (n=10/36) (Figure 2.1Q). 44 of the injected embryos were genotyped and, as expected, about one quarter (n=10) of these phenotypically normal embryos were homozygous for *apc*^{mcr}. This demonstrates that regulation of Wnt/β-catenin signaling is crucial for normal lateral line morphogenesis.

Misregulation of Wnt/β-catenin signaling in *apc*^{mcr} mutants is also apparent at the level of gene expression. *In situ* hybridization analysis revealed that expression of the Wnt/β-catenin target genes *lef1* and *axin2* is restricted to the leading zone of the wt primordium (Figure 2.1R,U,T,W). In contrast, 36 hpf *apc*^{mcr} mutants express these genes throughout the primordium (Figure 2. 1S,V). To investigate why *apc*^{mcr} primordia migrate normally until 27-29 hpf we performed *lef1* gene expression analyses and discovered that *lef1* is normally restricted until 27 hpf but expands into trailing cells over the next two hours coinciding with the onset of primordium stalling (Figure 2.2). Therefore, all subsequent analyses were performed at 32 hpf or older when the primordium has stalled in 100% of the mutants (n>500). The expression analyses and injection experiments suggest that primordium migration fails in *apc*^{mcr} mutant embryos due to ectopic activation

of Wnt/β-catenin signaling in the trailing zone of the primordium and not because of a failure of the Apc protein to associate with microtubules.

apc^{mcr} exhibit cell-autonomous migration defects but also affect neighboring wt cells

The gene expression analyses indicate that *apc*^{mcr} is required within the primordium. To investigate how *apc*^{mcr} mutant cells behave in an otherwise wt primordium, we transplanted red-labeled mutant cells into green *Tg(claudinb:gfp)* embryos during early gastrula stages and observed their behavior in mosaic primordia. In 7 of 9 mosaic embryos, *apc*^{mcr} mutant cells stopped migrating and were deposited ectopically (Figure 2.3A-C; arrows) between regularly spaced wt proneuromasts indicating that the *apc*^{mcr} mutation acts cell-autonomously. However, ectopic clusters always contained green wt cells demonstrating that *apc*^{mcr} mutant cells exert a noncell autonomous effect on neighboring wt cells, either via aberrant cell adhesion or aberrant cell signaling (Figure 2. 3B,C; n=7). In the two embryos in which *apc*^{mcr} mutant cells did not affect cell deposition, mutant cells were deposited early during migration, before the *apc*^{mcr} phenotype arises (data not shown). Wt cells transplanted into *apc*^{mcr} mutant embryos did not rescue the phenotype (Figure 2.3D-F; n=17). At around 29 hpf wt cells slowed down together with the surrounding *apc*^{mcr} cells. These data demonstrate that the *apc*^{mcr} phenotype is primordium autonomous and is not caused by defects in the muscle or skin of the mutants.

Wnt/β-catenin signaling regulates fgf signaling in the migrating primordium

Abrogation of the Fgf signaling pathway by application of the Fgf receptor inhibitor SU5402 immediately prior to primordium migration leads to primordium stalling strikingly similar to the phenotype observed in *apc*^{mcr} mutant embryos (Figure 2.4). We therefore asked whether Fgf and Wnt/β-catenin signaling interact during primordium migration. *fgf3* and *fgf10* are the only *fgf* ligands detectable in the primordium and are expressed in the leading zone of the wt primordium (Lecaudey et al., 2008; Nechiporuk and Raible, 2008), overlapping with the Wnt/β-catenin targets *lef1* and *axin2* (Figure 2.5A,E; 2.1R,U). To determine whether Wnt/β-catenin signaling regulates *fgf3* and *fgf10* expression in the leading zone of the primordium, we analyzed the expression patterns of these *fgf* ligands following changes in the level of Wnt/β-catenin pathway activation. *fgf3* and *fgf10* expression is upregulated in the entire primordium of *apc*^{mcr} mutant embryos (Figure 2.5B,F). Conversely, reducing Wnt/β-catenin signaling by overexpressing the inhibitor *dkk1* during primordium migration by heatshocking *Tg(hs:Dkk1)* transgenic embryos or by blocking β-Catenin-induced transcription by heatshocking *Tg(hsΔTCF:GFP)* embryos (data not shown) leads to strikingly decreased expression of *fgf3* and *fgf10* (Figure 2.5C,G) (Lewis et al., 2004; Stoick-Cooper et al., 2007). Importantly, Fgf signaling is not necessary for *fgf3* and *fgf10* expression in the primordium, as embryos treated with SU5402 show strong ectopic expression of these *fgf* ligands (Figure 2.5D,H). Therefore,

fgf3 and *fgf10* expression is regulated by Wnt/β-catenin signaling in the leading zone of the primordium.

To determine where Fgf signaling is active, we investigated the expression of the transcription factor *pea3*, a known target of the Fgf pathway (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Strikingly, *pea3* is excluded from the leading zone cells in migrating wt primordia, but is detected throughout the trailing end of the primordium (Figure 2.5I). These data indicate that Fgf ligands are able to act at a distance and that signaling may be inhibited in the leading zone. In contrast, 38 hpf *apc*^{mcr} mutant embryos express *pea3* throughout the primordium and in deposited cells. *pea3* is lost upon induction of *dkk1*, mirroring changes in *fgf3* and *fgf10* expression (Figure 2.5J,K). *pea3* expression is lost in SU5402 treated primordia, despite the dramatic upregulation of *fgf* ligands, confirming efficient inhibition of Fgf receptor activation (Figure 2.5D,H,L).

The striking absence of Fgf signaling activity from the leading cells that produce Fgf ligands in wt primordia could be caused by the presence of an inhibitor or the absence of Fgf receptors. Indeed, the only Fgf receptor present in the primordium, *fgfr1*, is expressed at very low levels in the leading zone of the wt primordium (Figure 2.6A,B; Lecaudey et al., 2008; Nechiporuk and Raible, 2008). In *apc*^{mcr} mutant embryos, *fgfr1* is also initially low in the leading zone of the primordium but becomes upregulated over time (Figure 2.6C,D). This upregulation can be explained by our finding that *fgfr1* is an Fgf pathway target in the primordium, as it is lost upon treatment with SU5402 (Figure 2.6E). We

hypothesize that stalling of the primordium exposes the leading zone to increasing levels of Fgf ligands over time, leading to upregulation of *fgfr1*. Another important mechanism to inhibit Fgf signaling is via the expression of the cytoplasmic membrane associated Fgf signaling inhibitor *sef (il17rd)* (Furthauer et al., 2002; Tsang et al., 2002). Since *sef* is co-expressed with *lef1* and *axin2* in the leading region of the primordium (Figure 2.5M), we tested whether Wnt/β-catenin signaling controls expression of this Fgf pathway inhibitor. Indeed, in addition to regulating *fgf* ligand expression, the Wnt/β-catenin signaling pathway also regulates expression of *sef*, as this gene is ectopically expressed in trailing cells in *apc*^{mcr} mutant primordia and is downregulated when Wnt/β-catenin signaling is depleted by induction of *dkk1* (Figure 2.5N,O). *sef* expression does not depend on Fgf signaling as it is ectopically expressed in trailing and deposited cells, even though it is downregulated in other regions of the embryo in SU5402 treated embryos (Figure 2.5P; 2.7A,C). Depletion of *sef* by morpholino injections causes expansion of the Fgf targets *pea3* and *dkk1* (see below) into the leading zone of the primordium accompanied by a primordium migration defect (Figure 2.5Q-V; 2.8)(Asai et al., 2006). Therefore, even though we cannot exclude the existence of additional mechanisms, *sef* plays an important role in inhibiting Fgf pathway activation in the leading zone, likely in combination with low *fgfr1* expression.

sprouty 4 (spry4) is another well-known Fgfr1 signal transduction inhibitor which is expressed in trailing cells of the wt primordium (Figure 2.9; (Furthauer et al., 2001)). In contrast to *sef*, *spry4* acts as a classical Fgf feedback attenuator as

it is lost upon SU5402 treatment, but is expanded in *apc*^{mcr} mutant primordia due to upregulation of Fgf signaling. (Figure 2.9B,C). Thus, *spry4* is not involved in inhibiting Fgf signaling in the leading zone.

To confirm the selectivity of SU5402 drug treatments, we compared treated embryos to a genetic knockdown of Fgf signaling via heatshock induction of *Tg(hs:dn-fgfr1)* which expresses a dominant negative Fgfr1 receptor ((Lee et al., 2005); (Figure 2.10A-D). A 1h heatshock induction of *Tg(hs:dn-fgfr1)* is similarly effective in eliminating *pea3* and *dkk1* and expanding *sef*, however, transgenic embryos begin to die within a few hours after the treatment (Figure 2.10E-G). To avoid possible interference from dying cells with the gene expression analyses we chose SU5402 to inhibit Fgf signaling.

Wnt/β-catenin mediated Fgf signaling is required in trailing cells for proneuromast development

A recent study by Millimaki et al. showed that Fgf signaling is required for the induction and maintenance of zebrafish ear and lateral line hair cells (Millimaki et al., 2007). Fgf signaling also controls proneuromast development in the lateral line by inducing radial epithelialization of support cells and regulating expression of the proneural transcription factor *atoh1* in patches containing cells fated to give rise to hair cells and support cells (Figure 2.11B; (Lecaudey et al., 2008; Nechiporuk and Raible, 2008; Millimaki et al., 2007).

Proneuromast development was analyzed in different experimental conditions. During wt proneuromast formation cells organize into rosettes that can be detected by cell shape changes and focal accumulation of Claudin-GFP

(Figure 2.11A). In 100% of *Tg(hs:Dkk1)* transgenic and SU5402 treated embryos no proneuromasts can be distinguished as Fgf signaling is disrupted in both conditions (Figure 2.11C,E). As expected, no *atoh1* expression is found in these primordia (Figure 2.11D,F). In contrast, in *apc*^{mcr} mutant embryos, in which Fgf signaling is active, proneuromasts show normal cell type specification (Figure 2.1K-N). Immediately after primordium stalling the leading zone in *apc*^{mcr} mutant primordia is still unpatterned, however, approximately five hours later a proneuromast begins to form in the leading zone (Figure 2.11G,H). As primordium stalling occurs before ectopic rosettogenesis in *apc*^{mcr} primordia, rosettogenesis in the leading zone is not the cause of the migration defect.

Rosettogenesis does not influence primordium migration

Our analysis of Wnt/β-catenin signaling revealed that primordia migrate in the absence of rosettes. Irrespective of when we abrogate Wnt/β-catenin (and secondarily Fgf signaling) by inducing *dkk1* primordia during migration, primordia continue to migrate to the tail tip in the absence of rosettogenesis. Primordium migration is evidenced by the presence of the lateral line nerve (Figure 2.11J,L; arrows; Figure 2.12) and timelapse imaging (not shown). We heatshocked embryos at 24hpf, after migration had started and shortly before the deposition of the first trunk neuromast (Figure 2.11I) and at 28hpf shortly after deposition of the second proneuromast (L2; Figure 2.11K). After each heatshock only one additional neuromast was deposited (Figure 2.11J-M). The effects of loss of Wnt/β-catenin signaling on rosettogenesis and migration were confirmed in *Tg(hsΔTCF:GFP)* embryos (Figure 2.13.) The finding that Wnt/β-catenin

signaling depleted primordia migrate, suggests that Wnt pathway activation in the leading zone is not required for migration but that ectopic expression of Wnt/β-catenin signaling in trailing cells impedes migration.

Fgf signaling inhibits Wnt/β-catenin signaling via *dkk1*

To further interrogate how Wnt/β-catenin and Fgf signaling interact we examined the effects of loss of Fgf signaling on Wnt/β-catenin pathway activation in migrating primordia. Pharmacological abrogation of the Fgf pathway during migration leads to ectopically expressed *lef1* and *axin2* in the trailing domain of the primordium (Figure 2.2R,U; 2.14A,B) similar to the phenotype observed in *apc*^{mcr} mutants (Figure 2.1S,V). These data indicate that Fgf signaling inhibits the Wnt/β-catenin pathway in the trailing zone of the migrating primordium. However, the ectopic activation of Fgfs in the *apc*^{mcr} mutant trailing zone, where β-catenin is constitutively active, is not able to suppress Wnt/β-catenin target genes. (Figure 2.1S,V; 2.5B,F). Therefore, the Fgf-induced inhibitory factor must be acting upstream of β-catenin. A likely candidate is *dkk1,* a diffusible inhibitor of the Wnt/β-catenin pathway (Niehrs, 2006). Indeed, *dkk1* is strongly expressed in a zone directly adjacent to the area where Wnt/β-catenin signaling is active and within the *pea3* expression domain marking the zone of Fgf pathway activation (Figure 2.14C). However, it is excluded from the most trailing cells. In contrast, *dkk1* is expressed throughout the lateral line of *apc*^{mcr} mutants (Figure 2.14D).

Although *dkk1* is classically thought to be a direct target of the Wnt/β-catenin pathway (Niehrs, 2006), its expression is abolished from migrating wt and

apc^{mcr} mutant primordia following Fgf pathway abrogation by SU5402 treatment or heat-shock induction of *Tg(hs:dn-fgfr1)* (Figure 2.14E,F; data not shown). This demonstrates that *dkk1* expression depends on Fgf signaling in the primordium. The proximity of *dkk1* expressing cells to the leading zone suggests that *dkk1* might also be controlled by Wnt signals, however this is not the case, as *dkk1* expression is completely lost in *apc*^{mcr} mutant embryos treated with SU5402 (Figure 2.14F). Thus, expansion of *dkk1* expression in *apc*^{mcr} mutant embryos is due to ectopic Fgf pathway activation and is only indirectly due to expansion of Wnt/β-catenin signaling. As expected, knockdown of *dkk1* by morpholino injection leads to expansion of Wnt/β-catenin target genes *lef1* and *axin2* in the primordium and causes primordium stalling (Figure 2.14G,H; data not shown; Seiliez et al., 2006). Dkk1 is thought to be a diffusible inhibitor, however, in the primordium it appears that Dkk1 does not diffuse very far, as *dkk1* and *lef1* expressing cells are situated right adjacent to one another (Figure 2.15). We have excluded that Dkk1 activity might be inhibited in leading zone cells as heat shock induction of *dkk1* is able to repress Wnt target activation (Figure 2.1T,W).

Taken together, these data imply a mechanism whereby Wnt/β-catenin pathway activation spatially restricts itself through Fgf-mediated induction of *dkk1*. This negative feedback loop between Wnt/β-catenin signaling and Fgf signaling ensures their mutually exclusive activation and maintains primordium polarity.

Localized Wnt/β-catenin signaling is necessary for asymmetric expression of chemokine receptors

Chemokine signaling is also crucial for primordium migration. Coordinated directional migration requires asymmetric expression of the chemokine receptors *cxcr4b* and *cxcr7b* in the primordium and expression of the ligand *sdf1a (cxcl12a)* in a narrow stripe along the horizontal myoseptum (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006; Li et al., 2004; Valentin et al., 2007). *sdf1a* is normally expressed in *apc*^{mcr} embryos, consistent with the tissue autonomous effect of *apc*^{mcr} revealed by transplantation experiments (Figure 2.3A-C; data not shown).

In wt primordia, *cxcr4b* is most highly expressed in the leading zone of the primordium, and is downregulated in trailing cells (Figure 2.16A) (Valentin et al., 2007). Conversely, *cxcr7b* is excluded from the leading zone but is strongly expressed in the trailing half of the primordium and in deposited cells (Figure 2.16B; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). In *apc*^{mcr} mutant embryos *cxcr4b* is ectopically expressed in trailing cells and *cxcr7b* expression is completely absent (Figure 2.16C,D). This initially led us to believe that either the Wnt/β-catenin or the Fgf signaling pathway initiates *cxcr4b* expression. However, Fgf signaling is not active in the leading zone of wt primordia and more importantly, pharmacological Fgf pathway inhibition with SU5402 also causes ectopic expression of *cxcr4b* and loss of *cxcr7b* (Figure 2.16E,F). Therefore, Fgf signaling does not regulate chemokine receptor expression in wt primordia. Instead, the misregulation of chemokine receptors in SU5402 treated embryos is

a secondary effect of ectopic activation of Wnt/β-catenin signaling. Interestingly, shutting down Wnt/β-catenin signaling by heatshock induction of *dkk1* does not affect *cxcr4b* expression, revealing that another factor activates *cxcr4b* expression in the leading zone (Figure 2.16G; data not shown). Since *cxcr4b* is ectopically expressed in *apc*^{mcr} mutant primordia, we conclude that the Wnt/β-catenin pathway is capable of inhibiting a repressor of *cxcr4b* in the most trailing cells. *cxcr7b*, on the other hand, expands into the leading zone when Wnt/β-catenin signaling is inhibited (Figure 2.16H). Therefore, Wnt/β-catenin signaling in the leading zone of the wt primordium localizes chemokine receptor expression by inhibiting expression of *cxcr7b*. Conversely, *cxcr4b* expression is inhibited in the trailing zone due to the absence of Wnt/β-catenin signaling.

Discussion

Our results demonstrate that the migrating primordium is stably patterned by interactions between the Wnt/β-catenin and Fgf signaling pathways. The Wnt/β-catenin pathway is activated only in leading zone cells of the primordium, where it stimulates the production of Fgf ligands and their inhibitor *sef* (Figure 2. 17A; red domain, yellow Fgf splotch). This results in activation of the Fgf pathway only in trailing cells (green domain; Figure 2.17A,B). Fgf pathway activation in trailing cells leads in turn to the production of *dkk1* that restricts activation of the Wnt/β-catenin pathway to the leading zone. Future experiments will address why *dkk1* is not expressed in all trailing cells.

Restriction of the Fgf and Wnt/β-catenin pathways is necessary for asymmetric expression of the chemokine receptors, *cxcr4b* and *cxcr7b* (Figure

2.17B). In contrast, *apc*^{mcr} mutant primordia are not polarized, displaying uniform activation of the Wnt/β-catenin and Fgf pathways throughout all cells (Figure 2.17B; 2.18A). Ectopic activation of these pathways causes upregulation of the inhibitors *sef* and *dkk1* throughout the primordium. Despite the upregulation of *sef*, Fgf signaling is still active, as revealed by the expression of the Fgf target *pea3* (Figure 2.5J; 2.17B). It seems likely that Sef abrogates Fgf signaling only in the presence of low levels of Fgfr1 as observed in wt primordia. Upregulation of *dkk1* has no consequence to Wnt/β-catenin signaling in *apc*^{mcr} embryos, as the Wnt pathway is constitutively activated by the mutation regardless of signaling events at the membrane. Interestingly, the pathway antagonists *sef* and *dkk1* are commonly thought to be feedback-induced by the Fgf and Wnt/β-catenin pathways, respectively (Chamorro et al., 2005; Furthauer et al., 2002). One exception is the Fgf inhibitor *dusp6* that is Wnt/β-catenin dependent during axis formation in zebrafish development (Tsang et al., 2004). We have discovered that in the lateral line primordium, *sef* expression is induced and maintained entirely by the Wnt/β-catenin pathway, whereas the Wnt/β-catenin inhibitor *dkk1* is regulated by the Fgf signaling pathway. This ensures that these two pathways are active in exclusive domains. These findings may warrant the reinvestigation of the hierarchy of these interactions in other developing organ systems.

<u>Regulation of chemokine receptors by the Wnt/β-catenin pathway</u>

The combination of gene expression changes and resulting phenotypes in the different experimental paradigms revealed that Wnt/β-catenin signaling in the leading zone restricts expression of *cxcr7b* to trailing cells (Figure 2.17). Ectopic activation of the Wnt/β-catenin pathway in *apc*^{mcr} or Fgf depleted embryos causes loss of *cxcr7b,* accompanied by ectopic expression of *cxcr4b* in trailing-most cells (Figure 2.18A,B). This result might suggest that Wnt/β-catenin signaling activates *cxcr4b.* However, inhibiting the Wnt/β-catenin pathway does not lead to a loss of *cxcr4b,* indicating that activation of *cxcr4b* expression in the leading zone occurs via an Fgf and Wnt/β-catenin independent mechanism (Figure 2.17). Furthermore, the observation that Wnt/β-catenin signaling is sufficient, but not necessary, for *cxcr4b* expression implies that ectopic Wnt/β-catenin signaling in trailing cells inhibits a transcriptional repressor of *cxcr4b* ('R' in Figure 2.17A). This repressor is independent of Fgf signaling, as *cxcr4b* is also repressed in trailing cells of SU5402-treated embryos. Our data suggest that the underlying cause of the migration defect in *apc*^{mcr} mutant and SU5402 treated embryos is the lack of *cxcr7b* and expansion of *cxcr4b* expression in trailing cells. Support for this conclusion also stems from time lapse analyses of *cxcr7b* morpholino injected embryos in which trailing cells tumble, but tip cells still extend normally toward the tail (Valentin et al., 2007).

Several models could explain how localized chemokine receptor expression controls directional migration. Based on experimental and genetic manipulations in which primordia migrated in either direction along the horizontal myoseptum, Sdf1a does not appear to be expressed in a gradient (Haas and

Gilmour, 2006; Smith et al., 1994; Stone, 1923). Therefore, it was suggested that polarized expression of *cxcr4b* and *cxcr7b* is likely responsible for setting up a Sdf1a gradient within the primordium (Dambly-Chaudiere et al., 2007; Valentin et al.). Since Sdf1a binds both Cxcr7b and Cxcr4b, the two receptors either have to bind Sdf1a with different affinities and/or initiate different intracellular signaling pathways in order to modulate the Sdf1a signal along the a-p axis of the primordium.

A recent study has elucidated a mechanism by which Sdf1 signaling is controlled by Cxcr4b and Cxcr7b during zebrafish primordial germ cell (PGC) migration (Boldajipour et al., 2008). Cxcr7b expressed in somatic tissue does not itself signal but acts as an Sdf1 sink, thus creating a Sdf1 gradient along which PGCs migrate. If Cxcr7b functions as an Sdf1a sink in the primordium, it could be necessary for establishing an Sdf1a gradient across the migrating primordium as suggested by Dambly-Chaudiere et al., 2007. Possibly, the sequestration of Sdf1a by *cxcr7b* expressing trailing cells, coupled with the sloping expression of *cxcr4b* enables individual cells within the primordium to orient towards the tail. In this model, apc^{mcr} and SU5402 treated primordia fail to migrate because all cells possess the same chemokine receptor expression and the ability to generate an Sdf1a protein gradient across any individual cell is lost. It is intriguing that tip cells continue to attempt directional migration long after trailing cells have begun tumbling in apc^{mcr} and SU5402 treated embryos. We believe that apc^{mcr} cells behaviorally and genetically resemble wt cells, as they express high levels of *cxcr4b* and lack *cxcr7*. As tip cells in apc^{mcr} mutant embryos cannot pull trailing

cells, this demonstrates that tip cells are not the only force-generating cells in the primordium, which is suported by live observations of individually labeled trailing cells (Haas and Gilmour, 2006).

Our data are consistent with Cxcr7b acting as an Sdf1a sink and we have demonstrated that Wnt pathway activation is necessary for restricting Cxcr7b to trailing cells where it could act to fine-tune the Sdf1a gradient. Alternatively, Cxcr7b could be activating an intracellular signaling pathway in trailing cells triggered by a secondary guidance signal produced by the tip cells to coordinate directed migration. This model was suggested by Haas and Gilmour and is based on their finding that a few wt tip cells can rescue the migration of *cxcr4b*-negative trailing cells (Haas and Gilmour, 2006). Future, detailed genetic and biochemical analysis of the characteristics and binding partners of *cxcr7b* will determine which model or combination of models is correct.

The Wnt/β-catenin/Fgf feedback loop coordinates primordium migration with proneuromast formation

Two studies investigating how loss of Fgf signaling affects proneuromast formation (rosettogenesis) found a tight correlation between rosettogenesis and migration. They concluded that normally formed rosettes are a prerequisite for primordium migration (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). However, our experiments demonstrate that the loss of Wnt/β-catenin signaling, secondarily causing the loss of Fgf signaling in *Tg(hs:Dkk1)* and *Tg(hsΔTCF:GFP)* embryos, does not cause primordium migration defects, even though it completely eliminates neuromast formation and deposition (movie S8,

Figure 2.11; 2.12; 2.13; 2.17B.) Therefore, neuromast formation and primordium migration are not interdependent. Our analyses revealed that the difference between SU5402 treated embryos and *Tg(hs:Dkk1)* embryos is that in SU5402 treated embryos Wnt/β-catenin signaling is still active, whereas in *Tg(hs:Dkk1)* embryos, both Fgf and Wnt/β-catenin signaling are abolished. Thus, the primary cause of migration defects in SU5402 treated embryos is not the lack of rosettes but the concurrent ectopic activation of Wnt/β-catenin signaling in trailing cells causing the loss of *cxcr7b* and ectopic *cxcr4b* expression (Figure 2.17B). Migration in *Tg(hs:Dkk1)* embryos is not affected, as *cxcr7b* is present in trailing cells and *cxcr4b* is unaffected. Comparing the effects of Wnt/β-catenin signaling manipulations on primordium behavior reveals that migration can occur in the absence of Wnt/β-catenin signaling but is disrupted in the presence of ectopic Wnt/β signaling in trailing cells.

Importantly, the comparison between apc^{mcr}, SU5402 treated and heat-shocked *Tg(hs:Dkk1)* embryos revealed that the Wnt/β-catenin/Fgf feedback loop in the primordium coordinates both migration and proneuromast formation by setting up primordium polarity via restriction of Wnt/β-catenin signaling to the leading zone, and by restriction of Fgf signaling to the trailing zone, where Fgf signaling initiates neurogenesis.

<u>Nature of β-catenin signaling pathway activation</u>

The identity of the ligand(s) that activate the β-catenin pathway in this system remains elusive. The best-known activators of the β-catenin signaling pathway are secreted Wnt ligands. However, this pathway can also be activated by other factors, such as ligands binding to G-protein coupled receptors (Sergey Shevtsov, 2006). Since the Sdf1a belongs to this class of proteins, this signaling pathway presented an interesting candidate for involvement in the regulation of β-catenin in the primordium. However, morpholino-knockdown of Sdf1a does not cause any changes in the expression of the β-catenin target gene *axin2* (Figure 2.19A,B). Importantly, depleting Dkk1 protein, which normally binds and inactivates the LRP family of Wnt receptors, causes upregulation of *axin2* and *lef1* (Figure 2.14G,H). Likewise, inhibiting Wnt signaling by heat shock induction of *dkk1* leads to the loss of these two genes (Figure 2.1T,W). Combined, these data reveal that the β-catenin signaling pathway must be activated by one or several canonical Wnt ligands. Wnt ligands could be locally produced within the leading zone of the primordium, they could be ubiquitously expressed in the environment, or they could be dynamically expressed surrounding the migrating leading zone of the primordium.

Collective cell migration in cancer

This work may have important implications for the spread of cancer cells. Several types of cancers invade surrounding tissue as collectives rather than as individual cells (Friedl, 2004; Friedl et al.,1995; 2004). The process of collective cell migration in cancer is still not well understood, but several studies indicate that clusters of cancer cells might be polarized similarly to the lateral line

primordium (Hegerfeldt et al., 2002; Wolf et al., 2007). It is likely that, just like in the lateral line primordium, opposing signaling pathways interact to set up this polarity.

CXCR4-SDF1 signaling is known to play a crucial role in the spread of many types of cancer and the work described here provides functional connections between this signaling cassette and the Wnt/β-catenin and Fgf signaling pathways, both of which are implicated in various steps of tumorigenesis (Polakis 2000; Kwabi-Addo, Ozen et al. 2004).

Since upregulation of Wnt signaling is commonly associated with tissue invasion and metastasis, it appears counterintuitive that *apc* mutant cells in the lateral line fail to migrate. However, APC mutant cells in the colon exhibit migration defects and fail to migrate from the base of the crypt toward the tip of the villus, where they would normally be shaved off into the lumen of the gut (Radtke and Clevers, 2005). CXCR4 is expressed in normal and transformed colon cells, but whether this compromised migration of APC mutant cells is caused by misregulation of chemokine signaling remains to be investigated (Jordan et al., 1999; Kim et al., 2005).

Irrespective of whether the signaling pathways employed to set up tissue polarity are identical between lateral line primordia and cancers, our findings provide an important conceptual framework for elucidating how interacting signaling pathways might control directional migration of clusters of cancer cells.

<u>Self organization within the migrating primordium</u>

The migrating primordium is a highly dynamic tissue. Cells are dividing along the whole axis of the primordium and clusters of cells constituting approximately 10-20% of the migrating tissue are dropped off from the trailing zone at regular intervals (Laurent Laguerre, 2005). Therefore, the identity of the trailing and leading zones must be continuously reinforced. In addition, the primordium encounters tissues with strikingly different positional identities during its head to tail migration. Because of this dynamism, a self-organized, tissue-autonomous signaling network is required to pattern the primordium. The model proposed here provides a mechanism whereby the primordium can be stably patterned, even though the relative location of individual cells is constantly changing by intercalary cell divisions and proneuromast deposition. The biological logic of the system, if not the molecules, may be found in other examples of morphogenesis involving highly dynamic organ anlagen.

Materials and methods

Fish strains

Time-lapse recordings were made using *Tg(Cldnb:lynGFP)* embryos, gift from D. Gilmour (Haas and Gilmour, 2006). Hair cells were visualized using *Tg(Brn3c:GAP43-GFP)*^{s356t} embryos (Xiao et al., 2005). *apc*^{mcr} mutants were a gift from H. Clevers (Hurlstone et al., 2003). *Tg(hs:Dkk1)* embryos were employed to inhibit Wnt/β-catenin signaling activation (Stoick-Cooper et al., 2007). *Tg(hsΔTCF:GFP)* embryos were used to confirm Wnt/β-catenin reduction phenotypes (Lewis et al., 2004). *Tg(hsp70l:dnfgfr1-EGFP)pd1* embryos were used to confirm the selectivity SU5402 treatments (Lee et al., 2005).

In-situ hybridization

Hybridization and staining were performed as described (Kopinke et al., 2006). In situ probes: *sef, fgf3* (Kudoh et al., 2001), *cxcr4b, cxcr7b* (Dambly-Chaudiere et al., 2007), *lef1* (Dorsky et al., 2003), *eya1* (Sahly et al., 1999), *pea3* (Munchberg et al., 1999), *klf4 (biklf)* (Kudoh et al., 2001), *fgf10* (Ng et al., 2002), *axin2* (gift from R. Dorsky), and *dkk1* (gift from C. Houart). Embryos were mounted in 100% glycerol. Images were taken with an Axiocam camera mounted on a Zeiss Axioskop 2 plus microscope.

SU5402 treatments

SU5402 (Calbiochem; gift from M. Brand; Mohammadi et al., 1997) was diluted to 25μM in E3 medium containing 1% DMSO. Dechorionated embryos were incubated from 18-20 hpf to 36-38 hpf. The effectiveness of Fgf signaling inhibition was confirmed by loss of *pea3* expression. Treated embryos were washed several times in E3 prior to fixation. Soaking embryos in 1% DMSO only did not cause a phenotype.

Heatshock induction of *dkk1, Δtcf* and *dnFgfR1*

Heterozygous fish were crossed to wt animals. Offspring were incubated at 42°C for 1 hour at various stages and fixed several hours later depending on the experiment. Fifty Percent of the embryos did not carry the transgene and served as a control. Effective inhibition of Wnt/β-catenin signaling was confirmed by loss of *axin2* and *lef1* expression from the primordia of heatshocked *dkk1* and

Δtcf embryos. Effective inhibition of FgfR1 was confirmed by loss of *pea3* expression.

Morpholino injections

MO-*dkk1*: 5’ GAGAGCATGGCGATGTGCATCATGT 3’ (Open Biosystems; Seiliez et al., 2006) 1nl of a 2mM solution was injected. This dose of morpholino yielded a spectrum of phenotypes identical to those reported, including reduction or loss of anterior sensory organs.

MO-*il17rd:*5' CGCAAGTCTCCGTGACCCAGCCATT 3' (Open Biosystems) (Asai et al., 2006). 1nl of a 3mM solution was injected. MO2-*cxcl12a:* 5' ATCACTTTGAGATCCATGTTTGCA 3'. (Open Biosystems)(David et al., 2002) 1nL of a 2mM solution was used. This dose recapitulated the published phenotype. Morpholinos were diluted in 0.1M KCl and 5% phenol red in water.

apc^{mcr} rescue experiment

apc^{mcr} rescue was performed as described (Hurlstone et al., 2003; Miller and Moon, 1997). mRNA was synthesized from a construct containing a central fragment of the human *APC* gene (a.a. 1020-2032) (Hurlstone et al., 2003; Miller and Moon, 1997). Injected 72hpf embryos were assayed for hair cell phenotypes by soaking them in 2mg/ml DASPEI ((2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide); Invitrogen) for 10 minutes (Whitfield et al., 1996). Significance of the rescue effect was tested using a test of difference in population proportions. To assay the rescue in individual mutants, individual

blastomeres of 8-16 cell-stage embryos were injected. This resulted in the production of *apc*^{mcr} homozygotes with unilateral rescue assayed by *eya1 in situ*.

Transplantation experiments

Tg(Cldnb:lynGFP) or *Tg(Cldnb:lynGFP);apc*^{mcr} donor embryos were injected with 5% Alexa568 and 3% lysine-fixable biotinylated-dextran (Invitrogen) at the one- to two- cell stage (diluted in 0.2 mM KCl). Cells were transplanted into the presumptive placode region (Kozlowski et al., 1997) of *Tg(Cldnb:lynGFP)* or *Tg(Cldnb:lynGFP);apc*^{mcr} between 30% and 50% epiboly. Host embryos were screened for lateral line clones and fixed at 36-40hpf. For *apc*^{mcr} to wt transplantations, donor embryos were raised to 36-40hpf for genotyping.

Phalloidin staining of rosettes

Embryos were fixed in 4% PFA for 2h at room temperature. Immediately after fixation embryos were incubated in phalloidin-Alexa568 (Invitrogen) diluted 1:20 in PBS + 1% Triton-X100 for 30 minutes. Stained embryos were visualized using a confocal microscope.

Time-lapse recordings

Embryos were anesthetized and mounted in 1.2% low melting point agarose in E3 medium. Recordings were made on an inverted Zeiss LSM5 confocal microscope with a climate chamber using a 10x or 20x objective and manipulated using ImageJ and apple QuickTime software.

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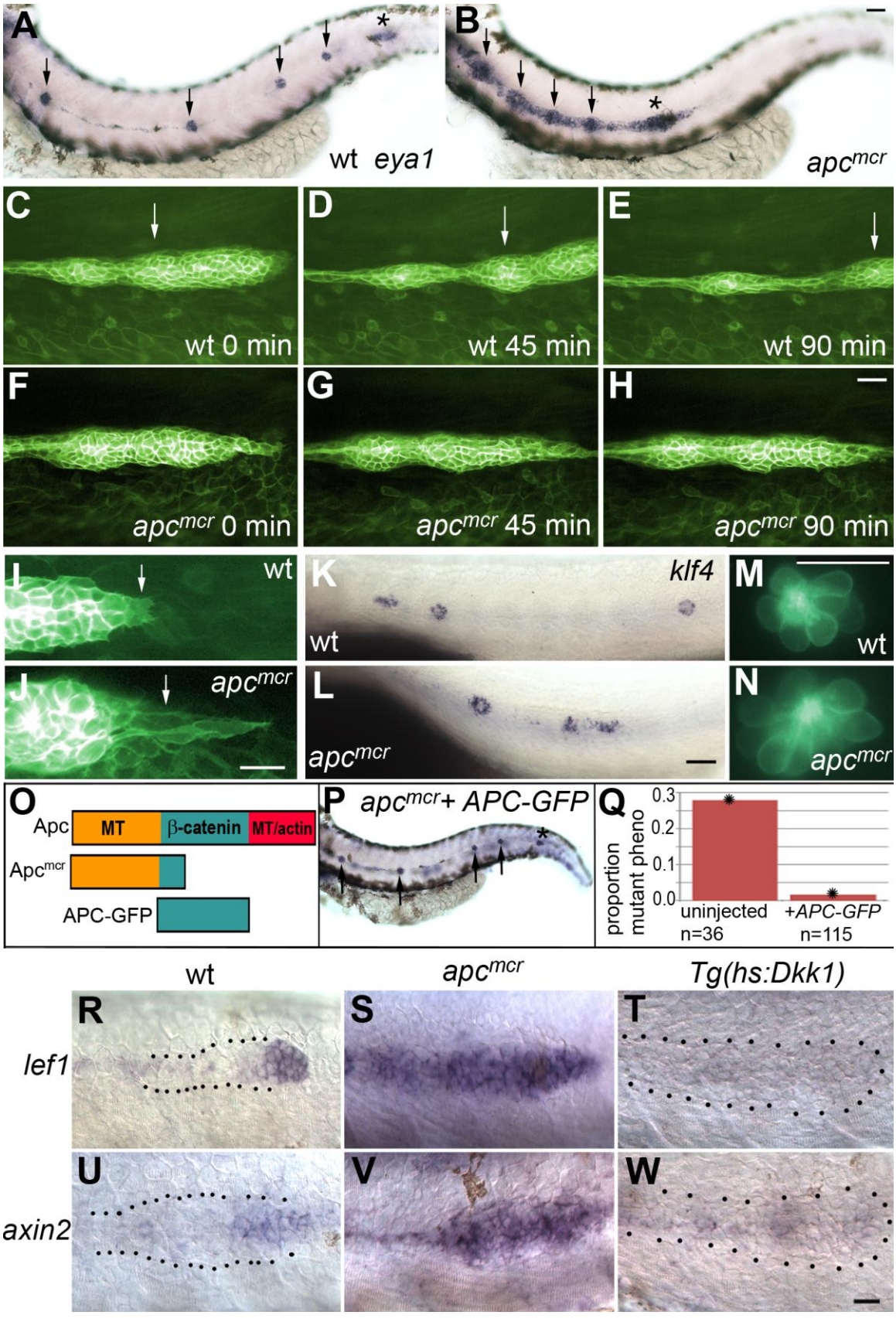
gure 2.1. Constitutive activation of Wnt/β-catenin signaling disrupts primordium migration but not cell type specification

Anterior is to the left in all figures. (A,B) *In situ* hybridization with the lateral line marker *eya1* of 36 hpf wt and *apc*^{mcr} mutant embryos. (A) In wt embryos the primordium (asterisk) has deposited neuromasts (arrows) and almost reached the tail tip. (B) In mutant embryos the primordium (asterisk) stopped migrating before reaching the tail. (C-E) Still images of a 90 min time-lapse recording of a normally migrating lateral line primordium in a *Tg(Cldnb:lynGFP)* embryo. (C) 34 hpf, time point 0 min. (D) after 45 min. (E) after 90 min. The trailing zone of the primordium (arrow) has almost left the field of view. (F-H) Still images of a 90 min time-lapse recording of a *Tg(Cldnb:lynGFP);apc*^{mcr} mutant primordium. (F) 34 hpf, 0 min. (G) after 45 min. (H) after 90 min. The primordium is not moving tailward. (I-J) Higher magnification images of tip cells (arrows) in migrating *Tg(Cldnb:lynGFP)* (I) and *Tg(Cldnb:lynGFP);apc*^{mcr} mutant primordia (J). Support (K,L) and hair cells (M,N) are specified in *apc*^{mcr} mutant embryos. *klf4 in situ* labels supporting cells in 36 hpf wt (K) and *apc*^{mcr} mutants (L). (M) Hair cells in *Tg(Brn3c:GAP43-GFP)*^{s356t} and (N) *Tg(Brn3c:GAP43-GFP)*^{s356t}*;apc*^{mcr} 72 hpf mutant embryos. (O) Simplified protein structure of wt Apc, Apc^{mcr} and APC-GFP. (P) *apc*^{mcr} embryo that was rescued by *APC-GFP* injection as revealed by *eya1 in situ*. Arrows indicate neuromasts, asterisk labels the primordium. (Q) Quantification of rescue effect. Rescue was found to be significant by a test of difference in population proportions ($P=2.3x10^{-}7$). (R,U) Wnt target genes *lef1* and *axin2* are expressed in the tip of 36 hpf wt primordia. (S,V) In *apc*^{mcr} mutant embryos *lef1* and *axin2* are expressed in the entire primordium and deposited cells. (T,W) *Tg(hs:Dkk1)* embryos, heatshocked at 20hpf, express no *lef1* or *axin2* by four hours post heat-shock. Scale bars in (A-L and R-W) equal 40µM. Scale bars in (M,N) equal 20µM.


A
wt eya1
B
apcmcr
C
wt 0 min
D
wt 45 min
E
wt 90 min
F
apcmcr 0 min
G
apcmcr 45 min
H
apcmcr 90 min
I
wt
J
apcmcr
K
klf4
wt
L
apcmcr
M
wt
N
apcmcr
O
Apc
MT
β-catenin
MT/actin
Apcmcr
APC-GFP
P
apcmcr+ APC-GFP
Q
proportion mutant pheno
0.3
0.2
0.1
0.0
uninjected
n=36
+APC-GFP
n=115
wt
apcmcr
Tg(hs:Dkk1)
lef1
R
S
T
axin2
U
V
W

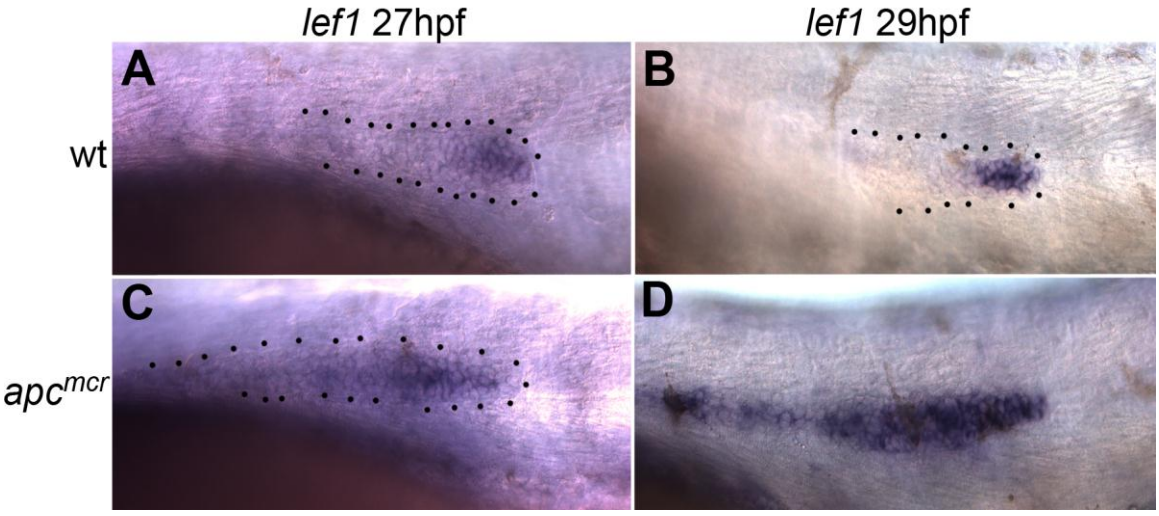


Figure 2.2 *apc*^{mcr} mutant primordia stall concomitantly with changes in gene expression between 27hpf and 29hpf

In wt primordia *lef1* is restricted to the leading zone of the primordium at 27hpf (A) and 29hpf (B). In *apc*^{mcr} mutant Frimordia *lef1* is initially restricted from trailing cells at 27hpf (C) but is fully expanded into the trailing zone by 29hpf (D).

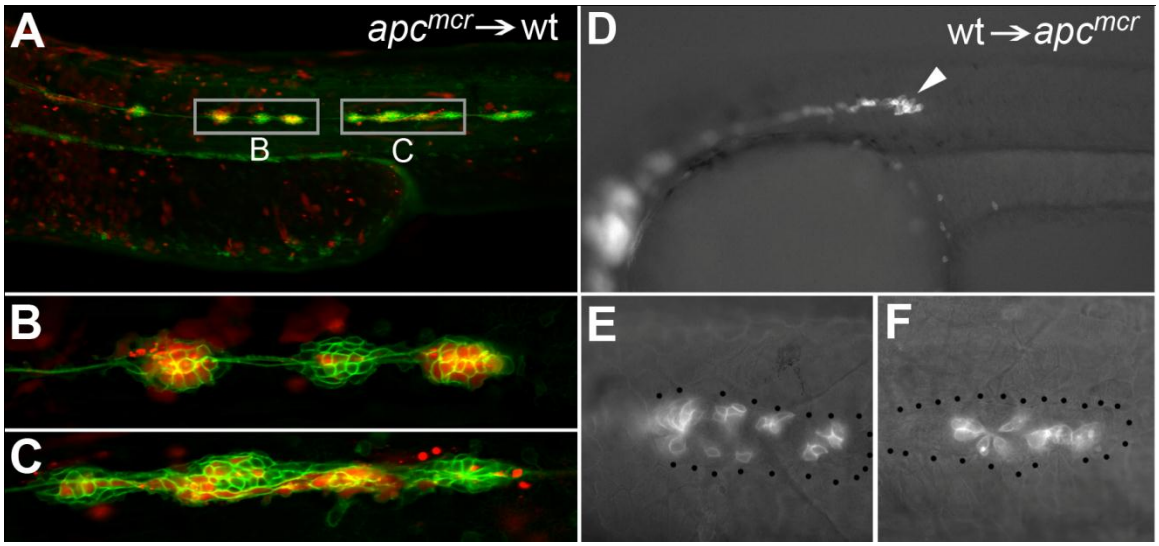


Figure 2.3. *apc*^{mcr} mutant cells display cell-autonomous defects and exert a non-cell autonomous effect on neighboring wt cells

(A) Mosaic embryo generated by transplanting red fluorescently labeled *apc*^{mcr} cells into *Tg(Cldnb:lynGFP)* embryos. (B,C) Higher magnification views of boxed areas in (A). (B) Red mutant cells are deposited and form proneuromasts in ectopic positions (arrows). (C) *apc*^{mcr} cells induce morphogenesis defects in neighboring green wt cells. (D) Overview of an embryo generated by transplanting fluorescently labeled wt cells into *apc*^{mcr} mutant embryos. (E,F) Higher magnification images of two additional wt to *apc*^{mcr} transplantations.

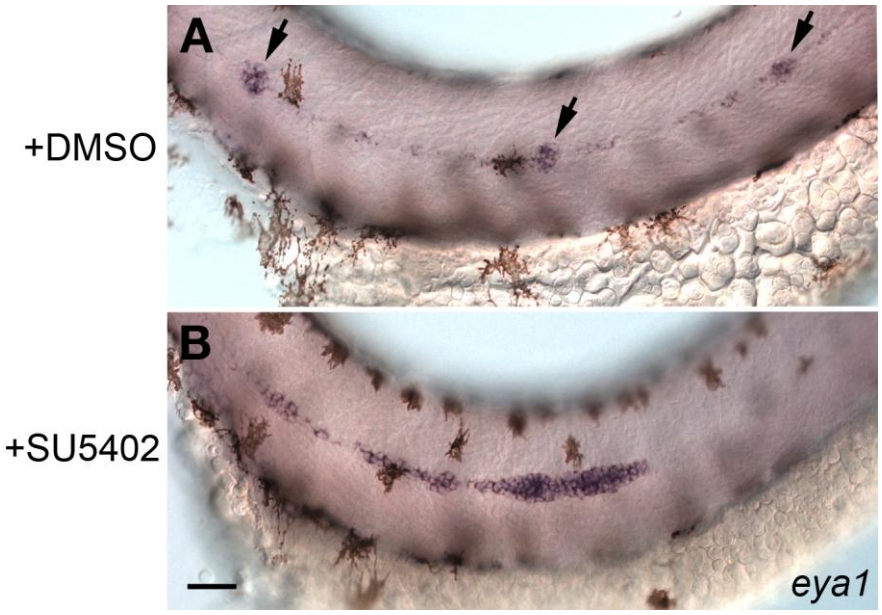
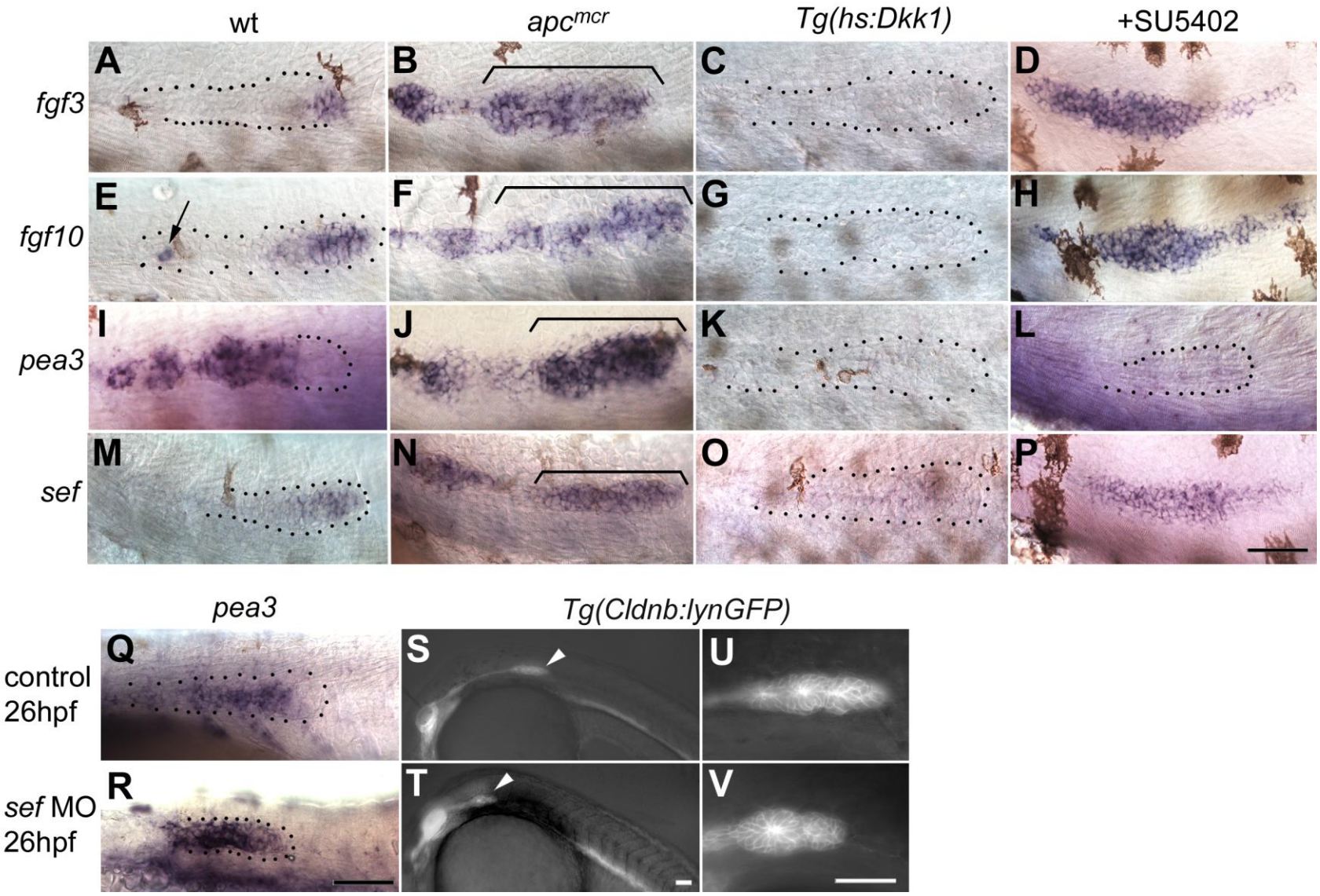


Figure 2.4. Abrogation of Fgf signaling leads to a primordium migration defect very similar to the defect observed in *apc*^{mcr} mutant embryos
(A) *In situ* hybridization with *eya1* at 38hpf. Primordium migration occurs normally and neuromasts are deposited (arrows) in wt embryos that have been incubated in DMSO between 20-38 hpf. (B) In wt embryos that have been incubated in DMSO and SU5402 the primordium stalls and fails to migrate to the tip of the tail. Scale bar represents 40uM.

Figure 2.5. Wnt/β-catenin signaling regulates Fgf signaling in the migrating primordium

fgf3 and *fgf10* are restricted to the leading zone of wt primordia (A,E) and are upregulated in *apc*^{mcr} mutant primordia (B,F). Their expression is lost in the absence of Wnt/β-catenin signaling (C,G). (D,H) *fgf3* and *fgf10* are upregulated in the absence of Fgf signaling. (I) *pea3* expression in wt primordia shows that Fgf signaling is only active in the trailing cells. (J) *pea3* is expanded in 38hpf *apc*^{mcr} mutants and lost in *Tg(hs:Dkk1)* embryos (K). (L) *pea3* expression is abolished by SU5402 treatment. The Fgf pathway inhibitor *sef* is expressed in the leading zone of wt primordia (M). *sef* expression is expanded in *apc*^{mcr} mutants (N) and abolished in *Tg(hs:Dkk1)* embryos (O). (P) *sef* expression does not require Fgf signaling as it is present in SU5402 treated primordia. Injection of *sef* morpholino (MO) disrupts primordium migration (S,T), although the primordia orient correctly towards the posterior (U,V). *pea3* expression expands into the leading region of *sef* morphant primordia (Q,R). Wt, *apc*^{mcr}, and SU5402 treated embryos were fixed between 32-36 hpf, *Tg(hs:Dkk1)* embryos were heatshocked at 26hpf and fixed at 32hpf, and *sef* morphant embryos were fixed at 26 hpf. Brackets in (B,F,G,N) indicate the primordium. Scale bars in (A-P and Q,R,U,V) equal 40μM. Scale bar for (S,T) equals 100uM.


wt
apc^mcr
Tg(hs:Dkk1)
+SU5402
fgf3
fgf10
pea3
sef
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
pea3
Tg(Cldnb:lynGFP)
control 26hpf
sef MO 26hpf
Q
R
S
T
U
V

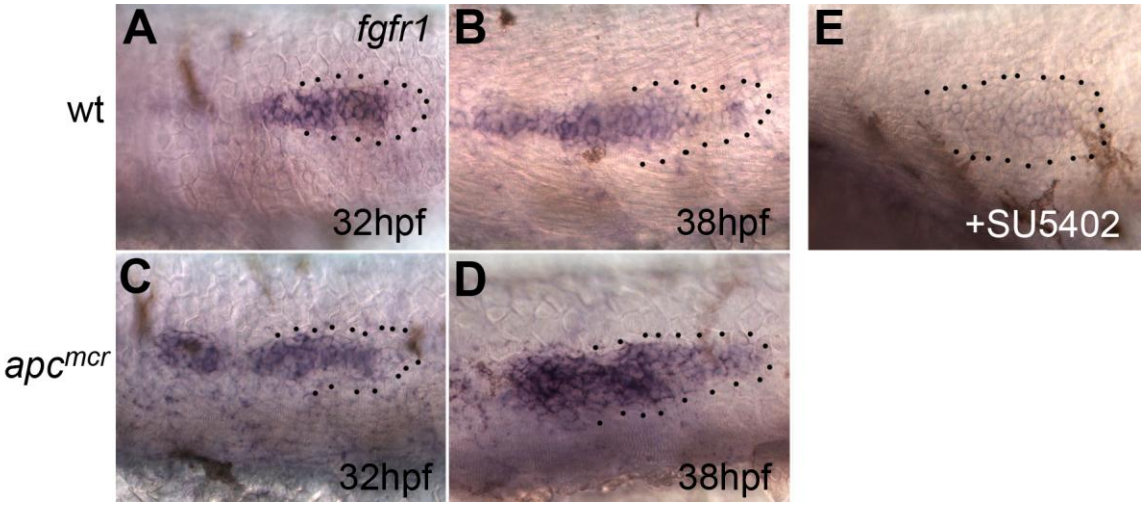


Figure 2.6. *fgfr1* is a feedback target of Fgf signaling in the primordium
fgfr1 expression is restricted from the leading zone throughout wt primordium migration (A,B). *fgfr1* is progressively upregulated in the leading zone of stalled *apc*^{mcr} mutant primordia between 32 and 38hpf (C,D). *fgfr1* expression is lost in embryos treated with SU5402 from 20-38hpf (E).

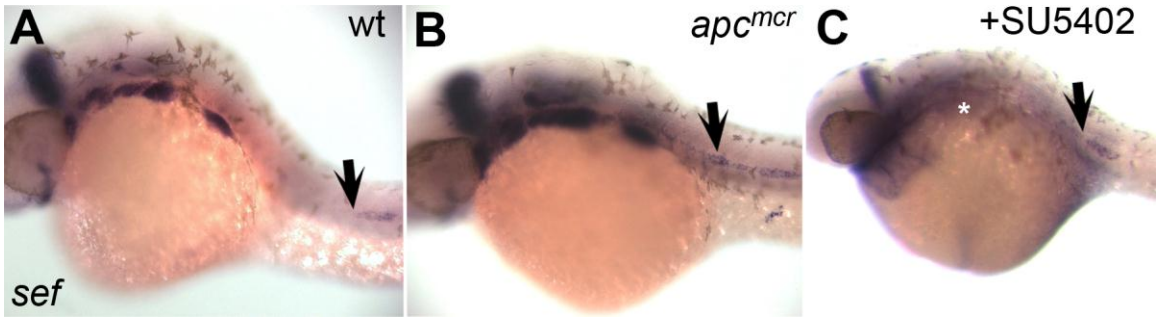


Figure 2.7. *sef* is a primordium-specific Wnt/β-catenin target
(A) In 32hpf wt embryos *sef* is absent in trailing cells in the primordium and in deposited cells (arrow). (B) 32 hpf *apc*^{mcr} mutants expresses *sef* ectopically in all lateral line cells (arrow), although global expression appears overtly normal. In Fgf inhibited 38hpf embryos *sef* is upregulated in trailing cells and is downregulated in other regions of the embryo, such as the pharyngeal arches (white asterisk) (C).

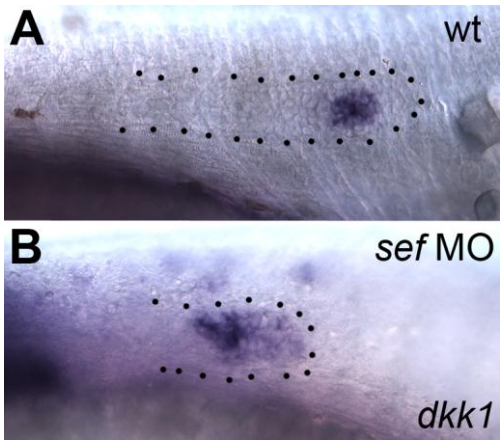


Figure 2.8. *sef* knockdown leads to ectopic expression of *dkk1* in the leading zone (A) *dkk1* is expressed in a region immediately adjacent to the leading zone in wt primordia. (B) In primordia of 26hpf *sef* morphant embryos *dkk1* is ectopically expressed in tip zone cells consistent with a role for *sef* in repressing Fgf signaling in these cells.

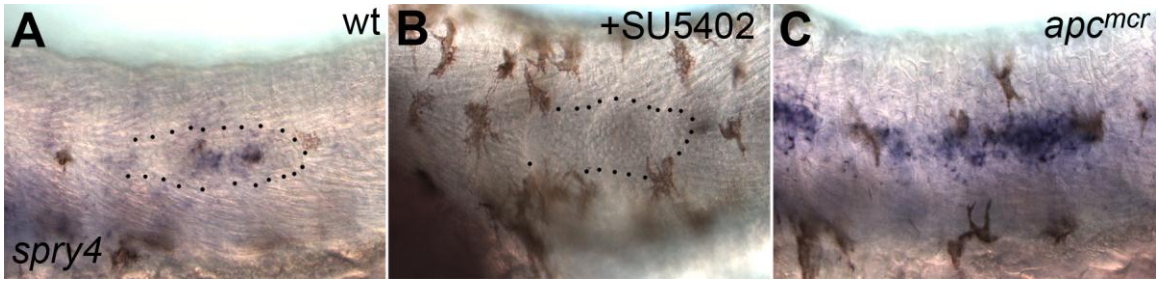


Figure 2.9. *spry4* is an Fgf target in the primordium

(A) *spry4* is expressed in the trailing zone of 32hpf wt primordia where Fgf signaling is active. (B) Expression is abolished in primordia exposed to the Fgf pathway inhibitor SU5402 from 20-38hpf. (C) Like other Fgf targets, *spry4* is expressed throughout the *apc*^{mcr} mutant primordium at 38hpf.

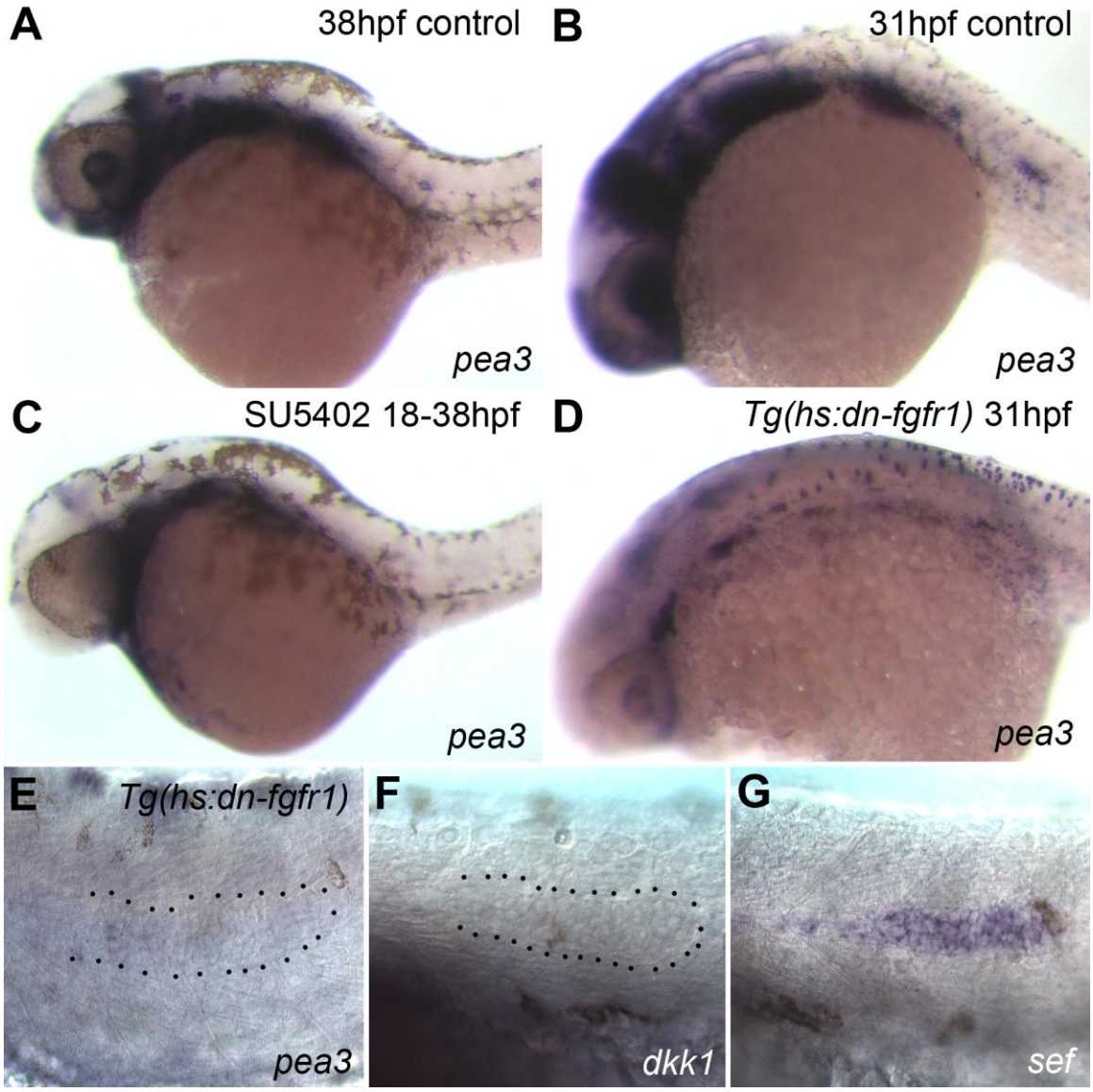
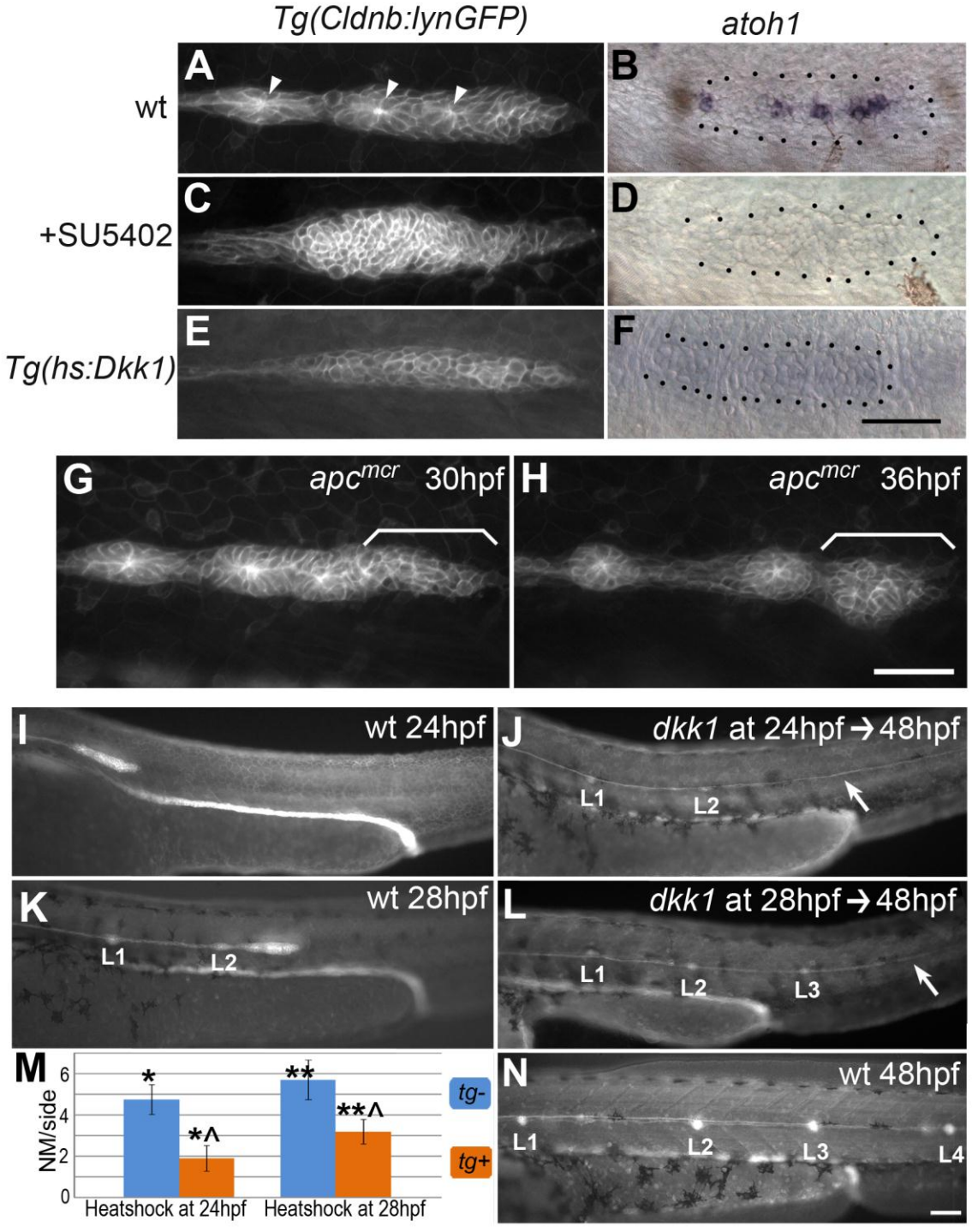


Figure 2.10. Induction of dominant negative Fgfr1 phenocopies Fgf inhibition via SU5402 treatment

(A,B) In wt embryos the Fgf target *pea3* is expressed in the lateral line as well as many other structures. Expression of *pea3* is strongly downregulated or completely abolished in embryos treated with SU5402 between 18-38hpf (C), and in 31hpf *Tg(hs:dn-fgfr1)* embryos at 4h post heat-shock (D). (E) Higher magnification image of the primordium of embryo shown in (D). Expression of the Fgf target *dkk1* (F) is also abolished 4 hours following heatshock induction of dn-Fgfr1. (G) The Fgf pathway repressor *sef* is ectopically expressed in the trailing edge 4 hours following induction.

Figure 2.11. Wnt/β-catenin mediated Fgf signaling is necessary for neurogenesis and rosette formation

(A) The trailing zone of wt primordia contains proneuromasts distinguishable by their rossette morphology, focal accumulation of Claudinb-GFP (arrows), and expression of the proneural gene *atoh1* (B) at 32hpf. (C) Loss of Fgf signaling by treatment with SU5402 between 20-38hpf causes loss of rosette formation and *atoh1* expression (D). (E) Similarly, abrogating expression of Fgf ligands by inhibiting Wnt/β-catenin via heat-shock induction of Dkk1 at 20hpf leads to a loss of rosettes and *atoh1* expression (F) at 28hpf. (G,H) Still images of movie S4. Ectopic rosette formation does not contribute to the apc^{mcr} migration phenotype, as stalled mutant primordia still have normally un-patterned leading zones (bracket in G). Ectopic rosette formation occurs in the leading zone at 5h after stalling (bracket in H). (I-N) *dkk1* disrupts neuromast (NM) deposition without affecting migration. (I) At 24hpf the wt primordium is migrating but no NM deposition has occurred. (J) *dkk1* induction at 24hpf leads to formation of a small L2 and complete loss of more posterior NMs without affecting migration as evidenced by the presence of the lateral line nerve (white arrow). (K) By 28hpf the wt primordium has deposited two NMs L1 and L2. (L) *dkk1* induction at 28hpf ablates the NMs posterior to L3. (M) Quantification of NM numbers for *dkk1* induction at 24hpf and 28hpf. On average, the primordium is able to deposit one additional NM after *dkk1* induction (orange bars). Data are shown as means ± SD (*P<<0.001, **P<<0.001, ^P<<0.001 Students T-test). (N) At 48hpf the wt primordium has deposited all posterior NMs.


Tg(Cldnb:lynGFP)
atoh1
wt
+SU5402
Tg(hs:Dkk1)
A
B
C
D
E
F
G
apcmcr 30hpf
H
apcmcr 36hpf
I
wt 24hpf
J
dkk1 at 24hpf→48hpf
L1
L2
K
wt 28hpf
L
dkk1 at 28hpf→48hpf
L3
M
NM/side
0
2
4
6
*
*^
**
**^
tg-
tg+
Heatshock at 24hpf
Heatshock at 28hpf
N
wt 48hpf
L4

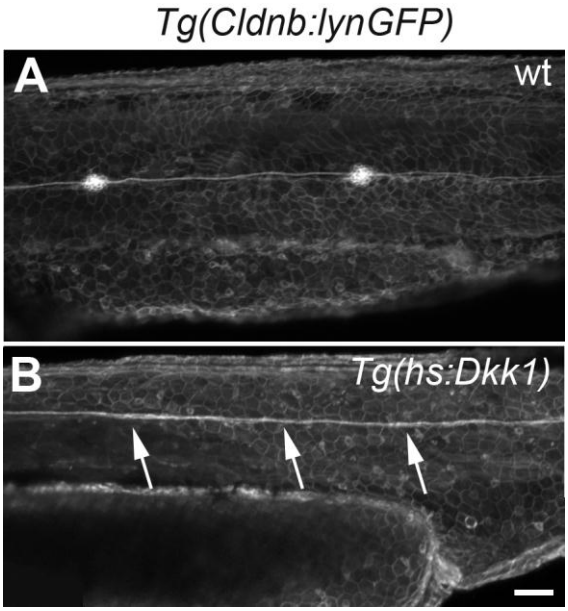


Figure 2.12. Loss of neuromasts in *Tg(hs:Dkk1)* is confirmed by confocal imaging
(A) In 48hpf *Tg(claudinb:gfp)* embryos, the primordia have migrated to the tail tip and deposited neuromasts. (B) Heatshocking *Tg(hs:Dkk1)* primordia at 26hpf leads to loss of posterior neuromasts at 48 hpf without affecting migration evidenced by the presence of the lateral line nerve (white arrows).

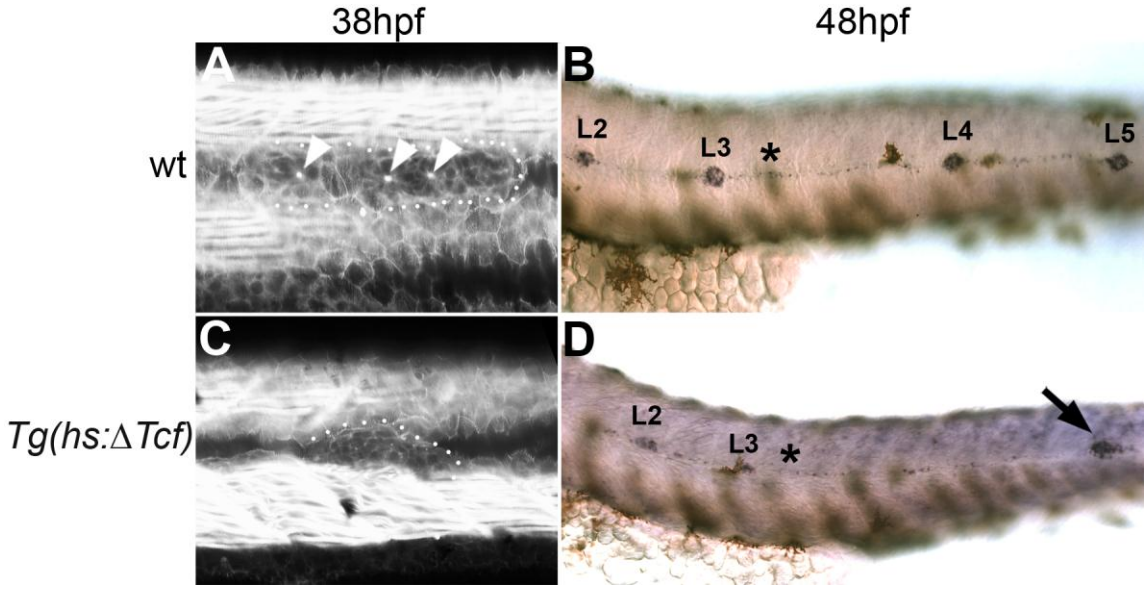


Figure 2.13. Rosette formation is lost in migrating *Tg(hsΔTCF:GFP)* primordia
(A) Rosettes can be visualized in 38hpf wt embryos by focal accumulation of F-actin revealed by phalloidin staining (arrowheads). (B) *eya1 in situ* shows that wt primordia have completed migration and deposited all posterior neuromasts by 48hpf (L2-L4). (C) Heatshock induction of the dominant repressor *ΔTCF:GFP* at 28hpf leads to complete loss of rosettes by 38hpf, as evidenced by phalloidin staining. At 28hpf the primordium is located just posterior to L3 (asterisk). (D) *eya1* in situ at 48hpf shows that heatshocked *Tg(hsΔTCF:GFP)* primordia continue to migrate following the loss of rosettes. Activation of *ΔTCF:GFP* is more detrimental to the general health of the embryos than activation of Dkk1. Therefore, heatshocked *Tg(hsΔTCF:GFP)* primordia stall at around the position of L5 (arrow) and the embryos eventually die.

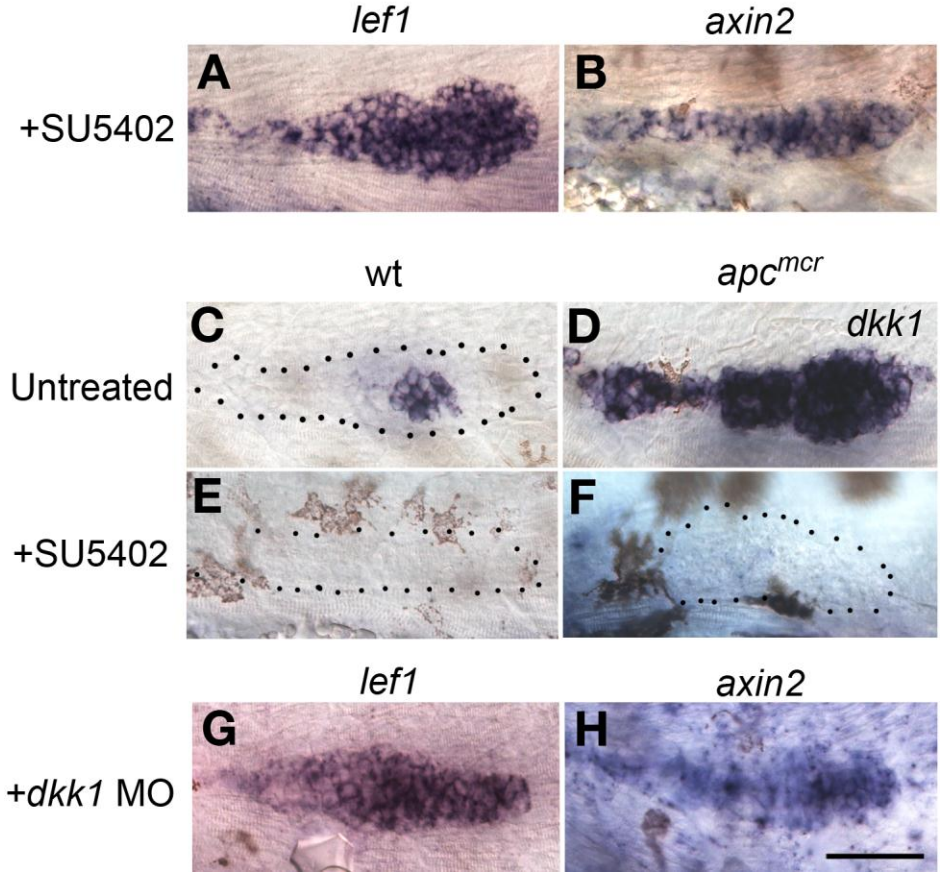


Figure 2.14. Fgf signaling inhibits Wnt/β-catenin signaling via induction of *dkk1*
(A,B) SU5402 treatment leads to ectopic induction of the Wnt/β-catenin signaling targets *lef1* and *axin2* in 36 hpf embryos. (C) In wt embryos, *dkk1* is expressed adjacent to the unpatterned tip of the primordium. (D) *dkk1* is a Fgf target, as it is highly upregulated in *apc*^{mcr} mutant primordia and is absent in Fgf signaling depleted wt (E), as well as in Fgf signaling depleted *apc*^{mcr} mutant primordia (F). (G,H) Morpholino knockdown of *dkk1* causes expansion of *lef1* and *axin2* similar to loss of Fgf signaling. Scale bars equal 40μM.

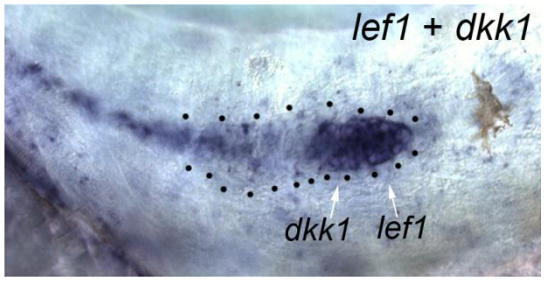


Figure 2.15. In wt embryos *dkk1* is expressed immediately adjacent to the leading zone in which Wnt/β-catenin signaling is active
Double *in situ* hybridization with *dkk1* and the Wnt/β-catenin target *lef1* at 32hpf reveals that Wnt/β-catenin signaling is not inhibited in cells immediately adjacent to *dkk1* expressing cells.

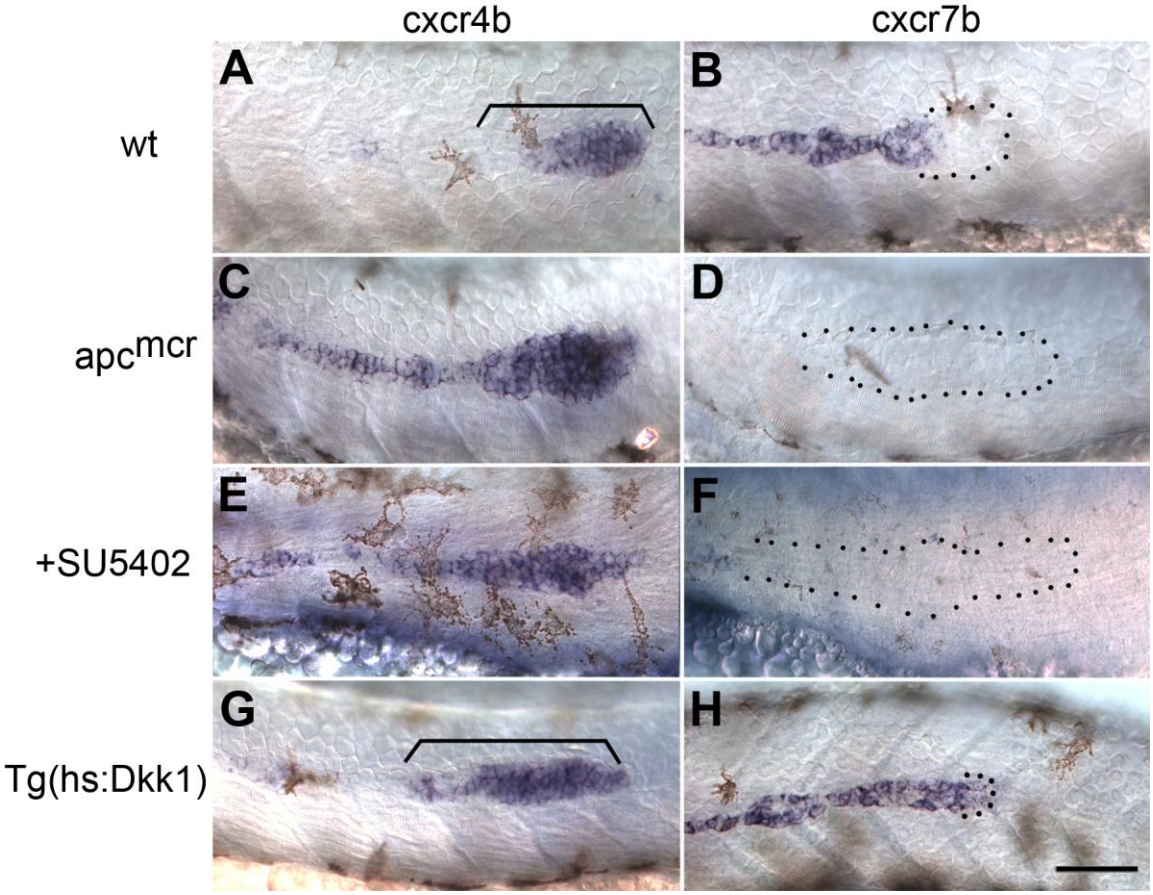
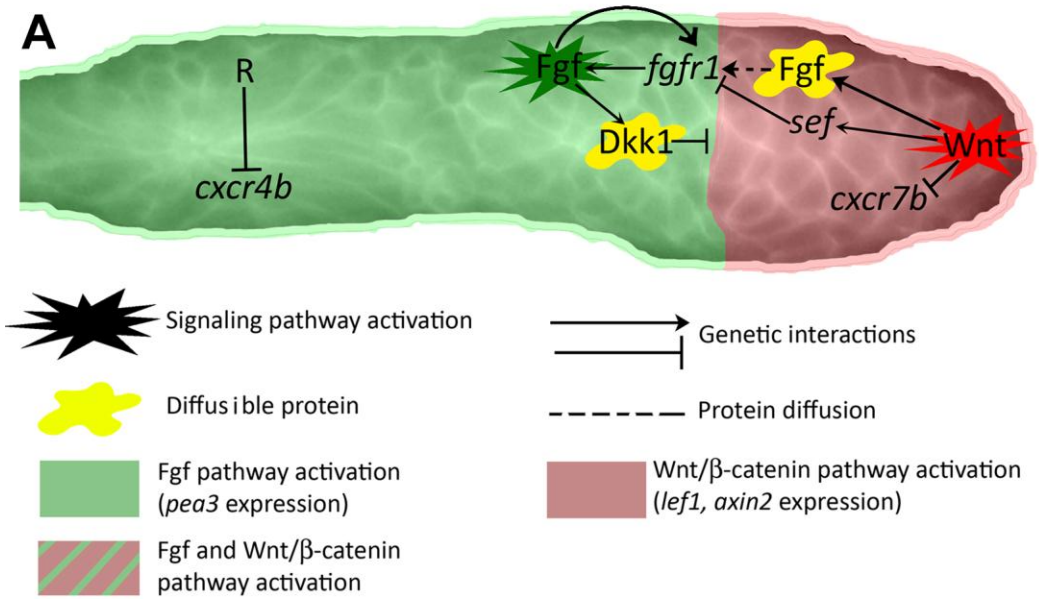


Figure 2.16. Localized Wnt/β-catenin signaling is necessary for asymmetric expression of chemokine receptors

(A) In 36 hpf wt embryos *cxcr4b* is restricted to the leading zone and *cxcr7b* is restricted to the trailing zone (B) of the migrating primordium. Expanded Wnt/β-catenin signaling in *apc*^{mcr} mutants and SU5402 treated embryos leads to expansion of *cxcr4b* (C,E) and loss of *cxcr7b* (D,F). (G) *cxcr4b* expression is not affected by a loss of Wnt/β-catenin signaling. (H) Loss of Wnt/β-catenin signaling leads to expansion of *cxcr7b* into the leading zone of the primordium. Scale bar equals 40μM.

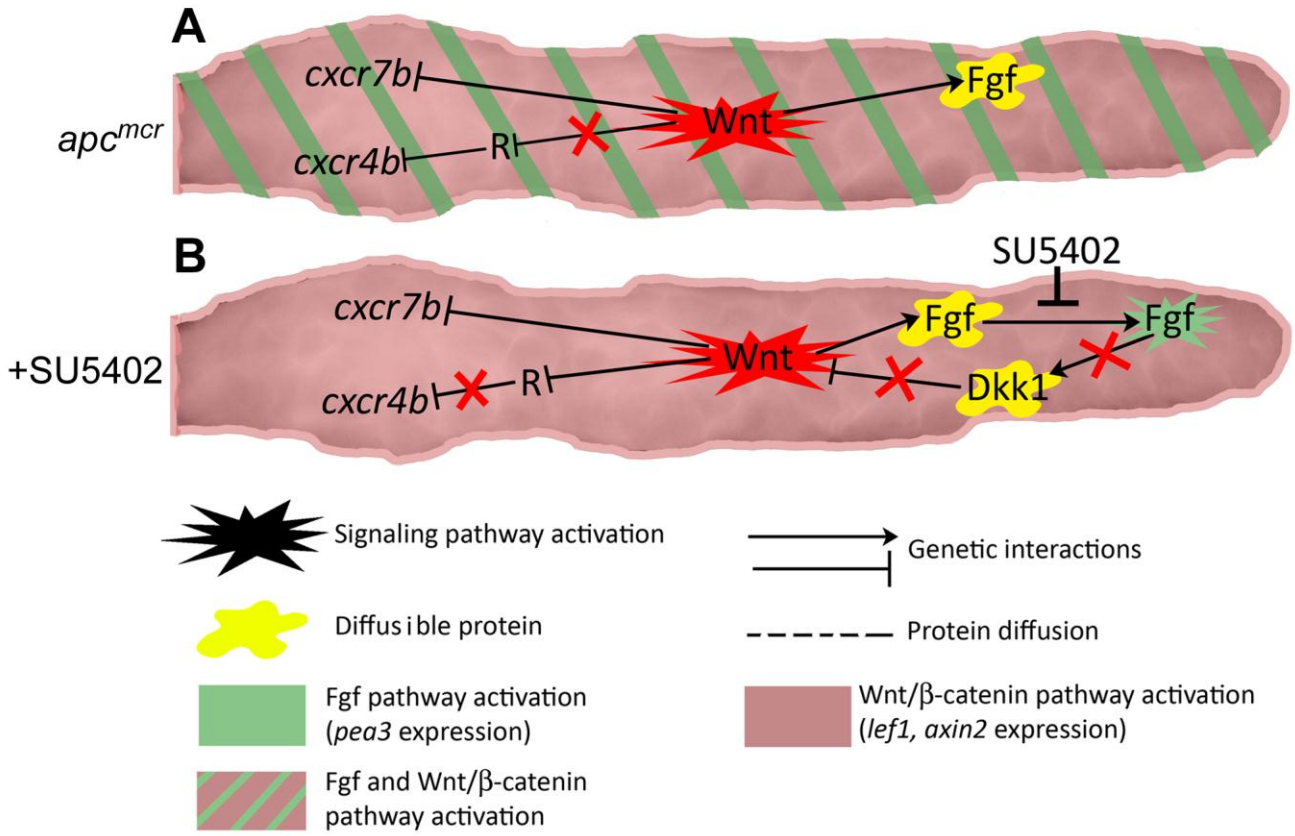
Figure 2.17. Schematic models of genetic interactions between signaling pathways and gene expression patterns in wt and manipulated primordia
(A) In wt primordia, Wnt/β-catenin pathway activation in the leading zone leads to Fgf pathway activation in the trailing zone. Exclusivity of these domains is maintained by the induction of *dkk1* by Fgf signaling in trailing cells and induction of *sef* by Wnt/β-catenin signaling in leading cells. *cxcr7b* expression in leading cells is inhibited by Wnt/β-catenin signaling, and *cxcr4b* expression is restricted from the trailing zone via the activity of an uncharacterized repressor (R) that is inhibited if Wnt/β-catenin signaling is active throughout the primordium. (B) Summary of gene expression patterns and associated phenotypes in the different experimental manipulations.



B	Wnt/β-catenin signaling	Fgf signaling	*cxcr4b* expression	*cxcr7b* expression	Proneuromast formation	Migration
wt					+++	+++
apc^{mcr}					+++	---
+SU5402 or +dnFgfR1					---	---
+Dkk1 or +ΔTcf					---	+++

Figure 2.18. Model of genetic interactions in Fgf-inhibited and *apc*^{mcr} mutant primordia

Both manipulations have the same effect on chemokine receptor expression. (A) In *apc*^{mcr} mutant primordia, both the Wnt/β-catenin and Fgf signaling pathways are active in all cells leading to global repression of *cxcr7b* and global de-repression of *cxcr4b*. (B) SU5402 inhibits activation of the Fgf signaling cascade, leading to loss of *dkk1* expression and expansion of Wnt/β-catenin signaling similar to the expansion caused by the *apc*^{mcr} mutation. Normal *cxcr4b* expression in these primordia implies the existence of a Wnt/Fgf independent activator. Red 'X's represent pathway disruptions. 'R' symbolizes an uncharacterized repressor.


A
apc^mcr
cxcr7b
cxcr4b
R
Wnt
Fgf
B
+SU5402
SU5402
Dkk1
Signaling pathway activation
Diffusible protein
Fgf pathway activation
(pea3 expression)
Fgf and Wnt/β-catenin
pathway activation
Genetic interactions
Protein diffusion
Wnt/β-catenin pathway activation
(lef1, axin2 expression)

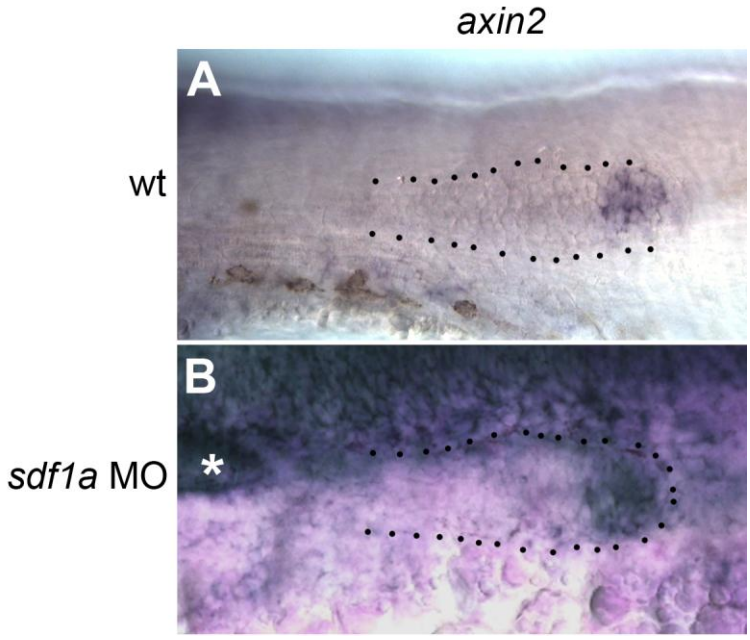


Figure 2.19. Sdf1a signaling is not necessary for local activation of the Wnt/β-catenin pathway in the primordium

In situ hybridization with *axin2* reveals that activation of the Wnt/β-catenin pathway in the leading zone occurs normally in 26hpf *sdf1a* morphant primordia (A,B). The *sdf1a* morphant primordium has not started to migrate and is still posterior to the otic capsule (white asteriks).

CHAPTER 3

MULTIPLE SIGNALING INTERACTIONS COORDINATE COLLECTIVE CELL MIGRATION *IN VIVO*

The following chapter is reprinted with permisson from:

Aman, A., Piotrowski, T. *Cell Adhesion and Migration,* 2009, 3:368-368.

Summary

Collective migration of adherent cohorts of cells is a common and crucial phenomenon during embryonic development and adult tissue homeostasis. The zebrafish posterior lateral line primordium has emerged as a powerful *in vivo* model to study collective migration due to its relative simplicity and accessibility. While it has become clear that chemokine signaling is the primary guidance system responsible for directing the primordium along its migratory path, it is not clear what mechanisms downstream of chemokine signaling coordinate migration of individual cells within the primordium. In this review, we summarize the cell signaling interactions that underlie collective migration of the primordium and discuss proposed mechanisms for the functions of chemokine signaling in this tissue.

Introduction

Cell migration is crucial for the embryonic development and homeostatic maintenance of multicellular animals. Although in many cases cells migrate as individuals, migration of adherent cellular clusters, sheets and chains is a fundamental morphogenetic process used to generate three-dimensional forms in the developing embryo. Collective cell migration underlies many important developmental events including gastrulation, the fusion of the two primary heart fields in vertebrate development, during blood vessel formation, wound healing and Drosophila tracheal development and border cell migration during oogenesis (Ghabrial and Krasnow, 2006; Martin and Parkhurst, 2004; Montell, 2006; Rorth, 2007; Schmidt et al., 2007; Solnica-Krezel, 2006; Trinh and Stainier, 2004).

Understanding collective migration also has clinical importance, as tumor invasion and metastasis often involves collective migration of cancer cells (Friedl, 2004; Rorth, 2007).

The zebrafish posterior lateral line (lateral line) has emerged as a powerful model for elucidating molecular genetic mechanisms that regulate collective cell migration *in vivo.* The lateral line is a sensory system found in fish and amphibians comprised of mechanosensory organs called neuromasts that contain sensory hair cells very similar to the hair cells that enable hearing in terrestrial vertebrates (Nicolson, 2005). The neuromasts of the lateral line are deposited along a stereotyped pathway by the migrating posterior lateral line placode (primordium), a cohesive cluster of over 100 cells that originates posterior to the ear and migrates along the embryonic trunk. During migration, cells in the trailing two thirds of the primordium organize into two or three garlic bulb-shaped rosettes. These rosettes are periodically deposited from the trailing edge of the primordium and subsequently mature to form mechanosensory neuromasts. Several excellent reviews describe the embryology of the lateral line in detail (Alain Ghysen, 2005; Dambly-Chaudière et al., 2003; Ghysen and Dambly-Chaudiere, 2004).

Chemokine signaling in the primordium

During the past seven years our knowledge about the regulation of primordium migration has increased significantly. The first clues into the molecular regulation of this process came in 2002 when David et al. discovered that the migrating primordium expresses the chemokine receptor *cxcr4b,* and that

the ligand *cxcl12a* (*sdf1a*) is expressed along the presumptive path of migration (David et al., 2002). Knocking down either gene caused a strong loss of migration. Intriguingly, it was shown that *cxcr4b* is most strongly expressed in the leading edge of the migrating primordium and is downregulated in cells about to be deposited from the trailing edge. Elegant gain of function studies showed that this signaling pathway is indeed the primary guidance system, as the primordium migrates toward an ectopic source of chemokine ligand (Li et al., 2004). In a different study it was demonstrated that in the absence of chemokine signaling, cells in the primordium are still quite motile, but lose their coordination and their directional collective migration (Haas and Gilmour, 2006). Subsequently, two groups independently discovered the presence of a second Cxcl12a-binding chemokine receptor Cxcr7b expressed in trailing cells of the primordium. Importantly, polarized expression of both *cxcr4b* and *cxcr7b* is crucial for normal migration (Figure 3.1A; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Despite rapid progress into understanding the regulation of collective migration of the primordium, key questions remain about how chemokine signaling directs collective migration of this tissue. Here we summarize proposed mechanisms of chemokine receptor mediated collective migration of the primordium and suggest approaches to resolve some lingering questions regarding the underlying molecular mechanisms.

With regard to Cxcr4b function, informative mosaic analyses have demonstrated that Cxcr4b is only required in a few tip cells, even though *cxcr4b* is expressed rather broadly in the primordium (Dark blue cells in Figure 3.1B;

Haas and Gilmour, 2006). Small clones of wildtype cells in otherwise *cxcr4b* deficient primordia completely rescue primordium migration. This approach also revealed that all cells in these mosaic primordia, including cells that presumably lack chemokine signaling, extend lamellapodia and actively migrate. These results lead to the proposal of a mechanism wherein only a few cells at the leading edge respond to Cxcl12a stimulation, causing the propagation of another signal or signals that polarize more trailing cells in the primordium (Figure 3.1B; green arrows). This second, intraprimordium signal could involve the propagation of a chemical signal similar to the production of chemotactic cAMP in the leading edge cells in *Dictyostelium* slugs (Dormann and Weijer, 2001). Alternatively, migration of *cxcr4b* expressing leading edge cells might stimulate the propagation of a mechanotactic signal similar to that observed in *in vitro* wound healing assays where tension on cells behind the leading edge is thought to stimulate ERK1/2 type MAP kinase signaling in more trailing cells (Lecaudey and Gilmour, 2006; Matsubayashi et al., 2004). At this point it is unresolved if leading cells migrate posteriorly because they respond to a Cxcl12a gradient, are intrinsically polarized or, are repelled by adjacent cells, as is the case in migrating neural crest cells (McDonald et al., 2008).

Cxcl12a belongs to a family of proteins known as chemokines, named for the fact that they are chemotactic cytokines. In most *in vitro* and *in vivo* contexts, these molecules allow the chemotaxis of cells up a gradient of ligand (Luster, 1998). It has been proposed that the primordium does not require a Cxcl12a gradient for directional migration, but that asymmetric expression of the

chemokine receptors Cxcr4b and Cxcr7b in the primordium direct migration along a homogenous stripe of Cxcl12a (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006; Valentin et al., 2007). While there is no obvious gradient of *cxcl12a* mRNA (David et al., 2002; Haas and Gilmour, 2006; Li et al., 2004), this finding does not preclude the possibility of an instructional gradient of Cxcl12a protein, especially given that chemokines have been shown to be post-transcriptionally regulated in other contexts (Boldajipour et al., 2008; Giraldez et al., 2006; Nakayama et al., 2007; Veldkamp et al., 2005).

The most recent experiment designed to evaluate the necessity of a chemokine gradient in primordium migration involved the *fused somites* (*fss*) mutant. *fss* homozygote embryos possess a truncated *cxcl12a* stripe that does not reach the tail tip. In these mutants, the primordium stalls upon reaching the end of the *cxcl12a* stripe. In the majority of cases, mutant primordia migrate ventrally towards the *cxcl12a* expressing pronephros. However some primordia make ‘U-turns,’ double backing on themselves dorsally, and migrating toward the head (Haas and Gilmour, 2006). These results demonstrate intrinsic polarity of the primordium but they do not rule out the presence of an instructive Cxcl12a gradient in wildtype embryos. As the mutant primordia reach the end of the *cxcl12a* stripe, it is conceivable that Cxcl12a protein continues to be produced by cells trailing of the primordium. This could lead to a reversal of the gradient with higher levels towards the head. Resolution of this issue will require assaying the concentrations of Cxcl12a protein along the anterior-posterior axis of *fss-/-* mutant embryos.

Regardless of whether an instructional gradient of Cxcl12a protein exists along the midline of the trunk, it has become abundantly clear that intrinsic polarity of chemokine receptors within the primordium is critical for coordinated collective migration (Aman and Piotrowski, 2008; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). A compelling model explaining the necessity of chemokine receptor asymmetry was originally proposed by Dambly-Chaudiere and coworkers (Dambly-Chaudiere et al., 2007). In this model, Cxcr7b in the trailing zone of the primordium does not signal but rather acts to sequester Cxcl12a protein, thereby establishing or amplifying a gradient of Cxcl12a protein across the tissue (Figure 3.1B). Elegant work on the role of Cxcr4b and Cxcr7b during zebrafish germ cell migration has since demonstrated that Cxcr7b can act as an Cxcl12a sink that binds and internalizes Cxcl12a protein generating protein gradients in extracellular space (Boldajipour et al., 2008). It is not clear whether Cxcr7b might also signal in response to Cxcl12a binding. CXCL12-CXCR7 interaction stimulates the AKT signaling pathway in prostate cancer cells, suggesting that CXCR7 can function as a signaling receptor in certain contexts (Wang et al., 2008).

Wnt/β-catenin and Fgf signaling in the primordium

Recent work from our laboratory has elucidated the complex cell signaling network underlying primordium polarity, including the asymmetric expression of *cxcr7b* in the migrating primordium (Aman and Piotrowski, 2008). The network is based on feedback interactions between the Wnt/β-catenin and Fgf pathways that restrict activation of these two signaling pathways to opposite poles of the

primordium. Wnt/β-catenin signaling is activated only in the first several rows of leader cells, where it induces the expression of secreted Fgf3 and Fgf10 ligands. Simultaneously, Wnt/β-catenin signaling upregulates the membrane tethered Fgf signaling inhibitor *sef* in leading cells. Therefore, Fgf pathway activation is inhibited in leading cells, even though these cells express Fgf ligands and results in the induction of Fgf target genes in trailing cells only. Fgf signaling in trailing cells, in turn, activates the potent Wnt/β-catenin signal inhibitor *dkk1*, which restricts Wnt/β-catenin pathway activation to cells occupying the leading portion of the primordium (Figure 3.1C; Aman and Piotrowski, 2008). By manipulating Wnt/β-catenin signaling using both gain and loss of function strategies and assaying chemokine receptor expression we discovered that Wnt/β-catenin activation represses *cxcr7b* expression in leading cells. Importantly, ectopic activation of Wnt/β-catenin signaling in the trailing portion of the primordium abolishes expression of *cxcr7b* in these cells. As a result, the primordium stalls similar to what is observed in *cxcr7b*-depleted embryos (Aman and Piotrowski, 2008; Dambly-Chaudiere et al., 2007; Valentin et al., 2007).

These studies also revealed that Wnt/β-catenin signaling not only regulates chemokine receptor expression but simultaneously influences proenruomast rosette morphogenesis. Neurogenesis and rosette formation depends on the Fgf-dependent expression of proneural genes and cell shape changes that drive rosette formation (Aman and Piotrowski, 2008; Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Wnt/β-catenin activation restricts Fgf-dependent neurogenesis to the trailing portion of the primordium and keeps the

leading portion unpatterned. Based on the analysis of Fgf depleted primordia which simultaneously lose rosettes and stop migrating it was postulated that rosette formation is indispensable for migration (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). However, our analysis of embryos in which Wnt/β-catenin and Fgf signaling was inhibited revealed that primordia migrate normally in the absence of rosettes and that stalling in Fgf depleted primordia is due to ectopic Wnt/β-catenin signaling and resulting loss of *cxcr7b* expression.

This Wnt/β-catenin-Fgf feedback mechanism maintains the stable asymmetric expression of chemokine receptors as the primordium migrates and deposits rosette clusters from the trailing edge (Aman and Piotrowski, 2008). Thus, interactions between the Wnt/β-catenin and Fgf pathways provide an elegant mechanism to couple forward migration with the periodic generation of sensory organs. A more complete understanding of collective migration of the primordium will require a mechanistic understanding of chemokine signaling in the developing lateral line.

Outlook

It is clearly important to resolve whether an instructional gradient of Cxcl12a protein exists along the horizontal myoseptum and how Cxcl12a protein distribution is affected by the passage of the primordium. We speculate that leading cells perceive a shallow Cxcl12a gradient that is amplified within the primordium by the intrinsic polarity of chemokine receptor expression. The isolation of a specific Cxcl12a antibody would allow the visualization of Cxcl12a protein distribution around wildtype primordia and primordia that have lost

chemokine receptor function. This approach would also aid in determining the role of Cxcr7b in shaping an extracellular Cxcl12a protein gradient around the primordium. It is also significant to establish the molecular functions of *cxcr7b* in the primordium. It is possible that, in addition to acting as a Cxcl12a sink, signaling via *cxcr7b* may also actively facilitate rosette deposition. This hypothesis is supported by the observation that *cxcr7b* is specifically expressed in cells fated to be deposited and that CXCL12-CXCR7 binding facilitates cell adhesion and survival *in vitro* (Burns et al., 2006; Dambly-Chaudiere et al., 2007; Valentin et al., 2007; Wang et al., 2008). Evaluating the role of Cxcr7b as a Cxcl12a sink can be accomplished using approaches similar to those employed for the analysis of germ cell migration in (Boldajipour et al., 2008). Such experiments include, for example, following the intracellular fate of tagged Cxcl12a and Cxcr7b protein. If Cxcr7b acts as a Cxcl12a sink, these two proteins should co-localize in intracellular vesicles and these vesicles should co-localize with lysosome markers. Evaluating a possible signaling role for Cxcr7b in the primordium will be more challenging, as little is known about how signal transduction downstream of Cxcl12a-Cxcr7b binding occurs. Structure/function approaches aimed at identifying domains in the Cxcr7b protein necessary for receptor internalization and/or signaling will be necessary to evaluate the role of Cxcr7b-dependent signaling in the primordium.

Resolution of these issues promises to yield a wealth of information on how collective cell migration is achieved *in vivo* that will deepen our

understanding of cellular mechanisms underlying morphogenesis and possibly also the spread of epithelial cancers.

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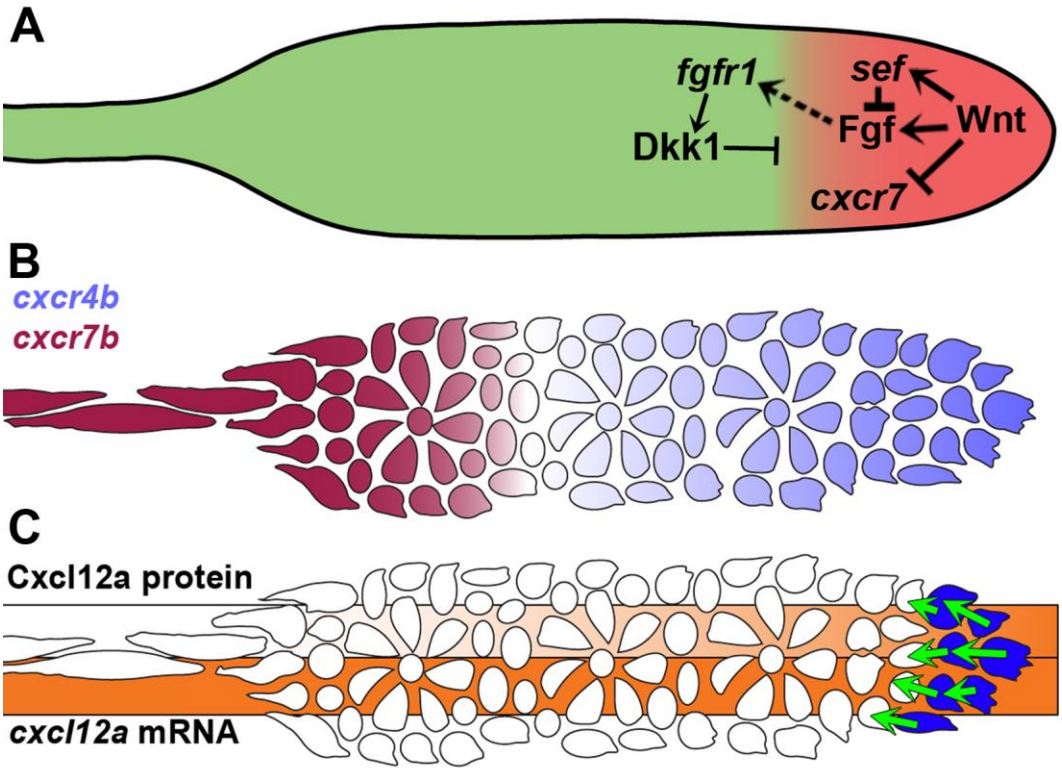

Figure 3.1. Schematic representation of signaling interactions underlying primordium collective cell migration

(A) Chemokine receptor expression in the primordium. *cxcr4b* (blue) is expressed most intensely by cells of the leading edge and is downregulated in trailing cells. *cxcr7b* (magenta) is expressed in trailing cells and cells that have been deposited. (B) Schematic of the hypothetical Cxcl12a protein gradient formed by *cxcr7b* expression in the trailing portion of the primordium. This gradient provides overall directionality to the cluster. Dark blue cells represent cells that must express *cxcr4b* for normal migration in mosaic embryos. Green arrows represent mechanical or chemical cues that coordinate the migration of individual cells within the primordium. (C) Primordium polarity is maintained by Wnt/β-catenin signaling. The leading zone (red) expresses Wnt/β-catenin target genes and the trailing zone (green) expresses Fgf target genes. Activation of the inhibitors *sef* and *dkk1* ensure mutual exclusivity of these domains. Solid lines represent genetic interactions. Dashed lines represent interactions involving diffusion of secreted factors. The posterior direction of migration is to the right in all diagrams.

CHAPTER 4

CELL MIGRATION DURING MORPHOGENESIS

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Summary

During development, functional structures must form with the correct three-dimensional geometry composed of the correct cell types. In many cases cell types are specified at locations distant to where they will ultimately reside for normal biological function. In other cases, cells are specified and must move to assume a specific geometry necessary for organ function. Cell migration is, therefore, crucial for the normal morphogenesis of animal body plans and organ systems. Although cell migration is necessary for normal development, abnormal cell migration during adult life underlies pathological states such as invasion and metastasis of cancer. Therefore, understanding the regulation of cell migration during development will not only give us a better understanding of morphogenesis, but also may provide insights that can be used to develop novel therapies aimed at preventing or treating metastatic cancer. In this review, we summarize and compare several *in vivo* model systems that have yielded insights into the regulation of morphogenic cell migration including migration of the lateral line primordium and primordial germ cells of zebrafish, border cell clusters in drosophila and angiogenic sprouts in the post-natal mouse retina.

Introduction

Cell migration is a widespread and complex process crucial to the morphogenesis of animal body plans and individual organ systems. Cells are specified in one region of the embryo during gastrulation and then migrate extensively before they reach their target. Additionally, reactivation of cell

migration processes underlies invasion and metastasis of human cancers, making the study of morphogenic cell movements clinically relevant.

Whether occurring during normal development or under pathological conditions, cells can either migrate individually or in groups. Individual cell migration has been noted in few, but nonetheless essential biological processes *in vivo*. Primordial germ cells (PGCs), leukocytes and hematopoietic stem cells, for example, migrate as individual cells (Friedl et al., 2001). In contrast, the number of contexts in which collective cell migration is known to occur has been increasing steadily in recent years. In *Drosophila*, this mode of cell migration is employed during border cell migration and tracheal development (Montell, 2003). In vertebrates, besides the widely studied collective migratory events of gastrulation and neural crest cell development, a key role for collective cell migration has been noted in vascular sprout and pronephros development, (De Smet et al., 2009; Teddy and Kulesa, 2004; Vasilyev et al., 2009) as well as in the development of the sensory lateral line in aquatic vertebrates (Ghysen and Dambly-Chaudiere, 2004). In all of these cases, groups of cells migrate as tightly associated epithelial sheets or clusters (*e.g.*, *Drosophila* border cells and zebrafish lateral line primordium), or they possess a mesenchymal character as during gastrulation and neural crest migration.

Because of its broad multicontextual and multiphylletic distribution, understanding cell migration in its various manifestations *in vivo* is likely to yield new insights into both the function and malfunction of key embryonic and postembryonic events. In this review, we will provide a succinct phenotypic

description of several important model systems utilized to study cell migration *in vivo*. More importantly, we will highlight, compare and integrate recent advances in our understanding of how cell migration is regulated in these varied model systems.

In vivo models of cell migration

Because of the many known manifestations of developmental cell migration, a broad spectrum of model systems has been utilized to functionally dissect this process. Cells can migrate either individually or collectively as cohesive clusters, sheets or chains. Below we summarize experimental results obtained from several of the most intensively studied examples of developmental cell migration and attempt to find general mechanisms shared between the different models. Special emphasis is placed on the regulation of three crucial steps of morphogenic cell migration: 1) How a cell or a group of cells first becomes motile and detaches from its tissue of origin. 2) How cells are guided toward target sites. 3) How cells ultimately stop migrating at the location where they are required for biological function (Figure 4.1). Regulation of these three steps is detailed for several prominent models of morphogenic migration and compared in an effort to find general principles illustrated by multiple model systems.

Primordial Germ Cells: A model of individual cell migration during development

In vivo single cell migration has been extensively analyzed and modeled by studying cells of the immune system. The migratory behaviors of

polymorphonuclear neutrophils from circulating blood to sites of infection or inflammation are well-known and have recently been reviewed (Cvejic et al., 2008). Migration of adult hematopoietic stem cells from the bone marrow into circulation and back to the marrow has also received its share of attention (Wright et al., 2001). Mechanistically, however, the study of germ cell migration during development has profoundly informed the field of cell migration. *Drosophila,* mouse and zebrafish are all powerful animal models for the mechanistic study of germ cell migration. In all of these model systems, primordial germ cells (PGCs) migrate long distances from their site of specification to the location of the prospective gonads (Figure 4.2A). While PGCs may migrate as clusters of cells in some species, in zebrafish PGCs do not migrate coordinately and no stable cell-cell contacts are established (Reichman-Fried et al., 2004). Zebrafish PGC migration is therefore considered a model for individual cell migration. *Drosophila* and mouse PGC migration has been comprehensively reviewed (Kunwar et al., 2006) and we will therefore focus on recent results obtained in the zebrafish.

In 2005, an *in vivo* study of zebrafish PGC migration and behavior characterized three phases of cell migration (Blaser et al., 2005). During phase one newly specified cells exhibit a simple morphology with no detectable protrusions (Figure 4.1A). In phase two the cells start to form protrusions in all directions but are still immotile (Figure 4.1B). The signals responsible for the transition from a newly formed, phase one, round PGC to a phase two cell with multiple cell protrusions are not known. However, knockdown of the gene

encoding the vertebrate-specific RNA-binding protein 'Dead end' blocks the competence of PGCs to become polarized and motile (Raz and Reichman-Fried, 2006; Weidinger et al., 2003). 'Dead end' suppresses the function of inhibitory miRNAs that normally suppress germ cell specific protein expression (Kedde et al., 2007). Unfortunately, it is not yet known how 'Dead end' influences cell motility.

Directional information in the form of a secreted guidance molecule is responsible for the transition into phase three, wherein the cells polarize their protrusions in the direction of migration and actively migrate toward their target (Figure 4.1C). Even though mouse, chick and zebrafish PGCs migrate through very different somatic tissues, they are all polarized and guided by Cxcl12a-Cxcr4b chemokine signaling (Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003; Stebler et al., 2004). Zebrafish PGCs express the chemokine receptor *cxcr4b* and surrounding somatic cells express variable levels of the ligand *cxcl12a* (Figure 4.2A). Cxcl12a-Cxcr4b signaling is thought to lead to asymmetric calcium signaling within a PGC that induces directional orientation of cellular extensions toward the higher concentration of chemokine (Blaser et al., 2006).

Interestingly, Blaser and colleagues revealed that protrusions form in a nondirected fashion long before the PGCs become motile and before they are able to respond to the chemoattractant Cxcl12a. Additionally, early expression of Cxcl12a is incapable of causing premature PGC migration (Blaser et al., 2005). Thus, protrusion formation is not chemokine-dependent. The PGC maturation

process occurs cell-autonomously, as older PGCs transplanted into younger host embryos start to migrate and reach the presumptive gonads before the endogenous PGCs do. These findings demonstrate that the regulatory interactions that lead to the acquisition of cellular protrusions necessary for migration can be distinct from the signals that ultimately guide a migrating cell toward its target. As we will see, this is a principle widely applicable to most, but not all, well studied examples of cell migration during development.

Recently, an elegant study by Boldajipour et al., has demonstrated that, in addition to *cxcr4b,* a second chemokine receptor, called *cxcr7b,* is required for germ cell migration (Boldajipour et al., 2008). Unlike *cxcr4b*, *cxcr7b* is not expressed by the migrating PGCs. Rather, this receptor is expressed broadly in somatic tissues surrounding the migrating cells. A series of experiments involving expression of functional fluorescent fusion proteins demonstrated that somatically expressed Cxcr7b binds and internalizes Cxcl12a, thereby removing it from extracellular space. This conclusion was confirmed by the demonstration that Cxcr7b expressing cells reduce the concentration of Cxcl12a in culture medium *in vitro*. Cxcr7b also limits the amount of Cxcl12a available for binding to Cxcr4b in PGCs *in vivo*, thus shaping a Cxcl12a protein gradient that is necessary to guide the cells toward the presumptive gonad. Consequently, knockdown of *cxcr7b* results in a dispersed pattern of PGCs, a phenotype similar to the one observed after overexpression of *cxcl12a.* Consistent with this role for somatically expressed *cxcr7b,* the *cxcr7b* loss of function phenotype can be partially rescued by a partial loss of *cxcl12a*. In summary, these experiments

revealed that in PGCs the Cxcl12a gradient is not generated by a standing source of passively diffusing ligand but rather via posttranslational regulation of Cxcl12a (internalization and destruction of Cxcl12a by surrounding cells).

PGCs stop migrating upon reaching a region of uniform Cxcl12a expression. Such a region exists at the site of the prospective gonad (Figure 4.1D; Doitsidou et al., 2002). Also, ectopic expression of Cxcl12a is capable of trapping PGCs in islands of high expression. Live imaging of stopping PGCs revealed that they still form protrusions as they stall. However, these protrusions lose polarity and further directed migration is inhibited (Reichman-Fried et al., 2004). Therefore, PGCs stop at their target destination due to a loss of directional information rather than a loss of motility. Uniform expression of guidance molecules is likely a general mechanism for stopping migrating cells at their target tissues, as will be seen in the discussion of other developmental models of migration below.

Border cells: a genetic model of collective migration

In the *Drosophila* egg chamber a group of cells migrates towards the posterior and then dorsal side of the oocyte where they contribute to the formation of the micropyle, which allows sperm entry and fertilization (Figure 4.2B). This group of cells is called the border cell cluster and consists of two cell types, the border and polar cells (Montell et al., 1992). The border cell cluster is specified at the anterior pole of the egg chamber (Figure 4.2B). To reach their final destination close to the oocyte, the 4-8 border cells and a pair of polar cells detach from the surrounding follicle cells and migrate posteriorly in between and

along 15 large nurse cells (Figure 4.2B). The polar cells are nonmotile and are surrounded by the border cells, which elaborate long cellular extensions and provide the force for motility. Because the border cell cluster stays cohesive throughout its migration, it is considered a model for collective cell migration. The power of *Drosophila* genetics has allowed screens for border cell migration-deficient mutants that revealed numerous genes affecting almost every step in border cell specification, onset of migration and directed migration (Montell, 2001; 2003; Rorth, 2002). Therefore, border cell migration is by far among the best-understood models of collective cell migration.

Border cell protrusions are triggered by secretion of the cytokine-like ligand ‘Unpaired’ (Upd) from polar cells (Figure 4.1B; Beccari et al., 2002; Ghiglione et al., 2002; Silver et al., 2005; Silver and Montell, 2001). Upd functions as a short range signal that leads to activation of the JAK/STAT signal transduction pathway and enables protrusion formation and detachment of follicle cells from the anterior pole of the egg chamber (Beccari et al., 2002; Silver and Montell, 2001). It has also recently been demonstrated that Notch signaling and core planar cell polarity (PCP) components are required for the acquisition of normal protrusive behavior (Bastock and Strutt, 2007; Prasad and Montell, 2007). Loss of Notch in border cells leads to fewer protrusions that are much longer lived suggesting defects in protrusion formation and dynamics (Prasad and Montell, 2007). Interfering with the core PCP pathway leads to a complete loss of actin rich protrusions (Bastock and Strutt, 2007). How these signaling pathways work together in regulating the complex and dynamic cell morphology that is

necessary for subsequent migration has not been investigated. However, based on the strength of the STAT loss of function phenotypes and the pleiotropy of the STAT pathway, it is tempting to hypothesize that JAK/STAT signaling may be acting upstream of Notch and PCP activity.

JAK/STAT signaling in border cells continues to be activated during migration by sustained secretion of Upd from the polar cells. This activation is required for the maintenance of protrusions and sustained motility (Silver et al., 2005). In rare cases where individual wild-type border cells lose contact with the polar cells, the detached border cells immediately lose protrusions and cease migration (Prasad and Montell, 2007). Interestingly, ectopic expression of Upd or mutations that activate the JAK/STAT pathway induce migration of usually non-motile follicle cells. These ectopically migrating follicle cells migrate individually or as differently sized clusters, suggesting that border cell cohesiveness is due to sustained requirement for Upd production by polar cells (Silver et al., 2005; Silver and Montell, 2001).

Detachment of the border cell cluster from follicle cells (Figure 4.1B) relies on the classical apico-basal polarity machinery defined by interactions between Par-1 at basolateral membranes and Par-3/aPKC at apical membranes. Loss of Par-1 function in border cells causes a strong defect in detachment and an associated loss of Par-3 localization. Additionally, overexpression of a non-localizable allele of Par-3 leads to the failure of border cell detachment (McDonald et al., 2008). The apically localized Par-3/aPKC complex is required for formation and stabilization of E-cadherin-based adherens junctions (Chen and

Macara, 2005; Hirose et al., 2002). During detachment, wild-type border cells reorganize E-cadherin from apically localized adherens junctions with neighboring follicle cells to broad baso-lateral domains between cells of the border cell cluster (McDonald et al., 2008). Mutations in the Par complex cause failure to reorganize these adherens junctions and therefore mutant cell are unable to detach. An interesting consequence of this reorganization is that the basal aspect of wildtype border cells in the migrating cluster faces the polar cells. Therefore, cells at the trailing edge of the cluster have the opposite orientation as cells at the leading edge (McDonald et al., 2008).

Additionally, although border cells deficient in Par-1 still extend protrusions, their directionality is lost and the undetached border cells extend more protrusions laterally along the follicle cells. Interestingly, the protrusion directionality defects were independent of Par-3 localization defects, as border cells expressing non-localizable Par-3 did not have such dramatic protrusion defects although they still fail to detach (McDonald et al., 2008). Therefore, Par-1 has at least two roles in early border cell migration: First, it must be present to polarize cells and reorganize adherens junctions enabling cluster detachment. Second, it is necessary for normal protrusive behavior via an unknown Par-3 independent mechanism.

For directed migration, border cell clusters do not utilize the chemokine signaling pathway but orient and migrate up a gradient of four functionally redundant growth factor ligands: Pvf1 (a PDGF/VEGF like factor), Spitz, Keren and Grk (Figure 4.1C; Duchek et al., 2001; McDonald et al., 2006; McDonald et

al., 2003). Pvf1, Grk, Spitz and Keren are produced in the oocyte at the time of migration and diffuse toward the anterior pole (Duchek et al., 2001; McDonald et al., 2006). These ligands bind to two partially redundant receptors, Pvr and Egfr, expressed in the border cells but not in the polar cells (Duchek et al., 2001; Wang et al., 2006).

The leading and trailing edges of individual cells, as well as cells occupying different positions within the cluster are exposed to different concentrations of chemoattractant growth factors. Two possible mechanisms exist by which the cluster orients in this gradient (Rorth, 2007). In the first mechanism individual cells mount different levels of nonlocalized signal transduction based on their position within the growth factor gradient. Comparisons between the levels of signaling in different cells then give the cluster directionality. In the second mechanism, individual cells respond independently and migrate towards a higher source of attractant by intracellular mechanisms that detect the highest level of attractant. In this scenario, asymmetry of attractant concentration across individual cells leads to localized accumulation of factors at the leading edge of the cell that are necessary for directional migration. Indeed, it has been shown that there is more intense growth factor signaling at the leading edges of individual cells in the border cell cluster and that disrupting this localization coincides with migration defects (Jekely et al., 2005). Of course, these two mechanisms are not mutually exclusive and may both be operating at the same time. For instance, asymmetric receptor activation across individual cells may polarize these cells toward the

higher concentration of attractant and, simultaneously, differences in between the total levels of signaling within cells occupying different positions of the cluster might be polarizing the entire migrating tissue.

Live imaging experiments to observe the behavior of migrating border cells has allowed evaluation of these mechanisms. If cluster polarity is important for directional migration then this polarity should be stable and the border cells will not shift relative to other cells in the cluster during migration. On the other hand, if all the cells of the cluster are guided independently they should be free to exchange position within the cluster as it migrates. Early in migration, cells at the leading edge of the cluster maintain their position and extend much longer protrusions than other cells (Bianco et al., 2007). During this phase loss of growth factor receptors leads to increased protrusions from cells at the rear of the cluster (Prasad and Montell, 2007). Because the border cells are polarized outward with their baso-lateral domains facing the centrally located polar cells, protrusions from cells at the trailing edge of the cluster generate force in the opposite direction of normal migration impeding forward progress of the cluster. Therefore, some presently unknown growth factor-dependent mechanism is necessary to limit the protrusiveness of trailing edge cells (Prasad and Montell, 2007). Because cells do not exchange positions within the cluster and cells at the leading edge exhibit different behavior from those at the trailing edge, cluster polarity appears to be important during this early phase of border cell migration.

As migration continues, however, cells in the cluster begin to constantly exchange positions (Bianco et al., 2007). Therefore, stable cluster polarity plays

a minor role in guiding directional migration at these later stages. As the cluster enters a region of higher growth factor concentration, an individual cell chemotaxis mechanism becomes more prevalent. Supportive evidence for this finding is that slight overexpression of growth factor attractant that presumably preserves its gradient speeds the transition to individual cell chemotaxis. Availability of more growth factor causes individual migrating cells to exchange positions within the cluster prematurely (Bianco et al., 2007).

Upon reaching the oocyte, border cell protrusions become nonpolarized and migration ceases, although formation and extension of undirected extensions may go on for some time afterwards (Prasad and Montell, 2007). Similar to PGCs, this stalling is likely due to the cells occupying a region of uniform chemoattractant and therefore losing guidance information. Consistent with this interpretation, drastic overexpression of growth factor chemoattractants throughout the egg chamber that abolishes the gradient also abolishes directional migration, even though the cells are still protrusive but unpolarized (McDonald et al., 2006).

The posterior lateral line: a vertebrate model of collective migration

Cranial placodes are transient embryonic structures that give rise to a variety of sensory organs and ganglia in nonmammalian and mammalian vertebrates (Schlosser, 2006; Schlosser and Northcutt, 2000). Cranial placodes are specified in the vertebrate head in a pan-placodal horseshoe-shaped region (Schlosser, 2006). Although placodal tissues are specified in a single broad

location, they eventually occupy locations distributed along the head and, in the case of the posterior lateral line placode of aquatic vertebrates, along the entire anterior-posterior axis of the animal. This distribution is achieved by subdivision of the pan-placodal field into separate placodes and subsequent migration of placodally-derived cells to the locations in which they are required to differentiate in order to establish normal sensory or secretory function. The development of the sensory lateral line system of aquatic vertebrates has emerged as a powerful model to investigate placode migration. The lateral line system is composed of a series of mechanosensory organs (neuromasts) in the skin of the animal (Figure 4.3A, yellow spots). Neuromasts contain hair cells that sense water motion and enable the animal to orient, socialize and forage. All cells of the lateral line are derived from migrating cranial placodes (Figure 4.3B). Primitive ray-finned fish, such as the actinopterygian *Polypterus*, possess six embryonic lateral line placodes that give rise to several lines on the head and the trunk (Figure 4.3C; Piotrowski and Northcutt, 1996). In teleosts, such as the zebrafish the placodal field subdivides into only an anterior and a posterior lateral line placode. The posterior lateral line placode (hereafter referred to as the primordium) migrates from behind the ear to the tail tip as a compact cluster of approximately 100 cells, periodically depositing clusters of cells that subsequently form sensory organs (Figure 4.3D; Metcalfe et al., 1985). All cells of the migrating cluster extend protrusions in the direction of migration. Extensions from cells occupying the leading edge of the cluster are readily apparent, while extensions from more

trailing cells extend underneath the cells in front of them and require mosaic labeling to observe (Haas and Gilmour, 2006).

In contrast to *Drosophila* border cells, the posterior lateral line placode differs in one important aspect of how it migrates collectively. The posterior lateral line placode moves as a tight cluster of cells that rarely exchange relative positions during migration, whereas border cells adhere only loosely to each other and exchange positions frequently. Here we focus on the significant progress that has been made in our understanding of collectively migrating cells using the zebrafish posterior lateral line cells as a model system.

The posterior lateral line primordium is specified at the extreme posterior tip of the pan-placodal region in zebrafish embryos (Kozlowski et al., 1997). Cells in the premigratory primordium begin extending protrusions in all directions and exhibit tumbling motility by 18 hours postfertilization (hpf; AA & TP, unpublished observations). Around 22 hpf protrusions of primordium cells become oriented and the cluster begins migrating posteriorly (Sapede et al., 2002). It is presently not known what triggers the onset of motility in the posterior lateral line primordium. The premigratory primordium begins to express *cxcr4b* at the same time when cells begin to tumble (18 hpf), which suggests that chemokine signaling might trigger the formation of protrusions. However, embryos in which the *cxcl12a* guidance molecule (see below) or its receptors in the primordium are mutated or inhibited, still possess protrusions and are quite capable of undirected tumbling motility (Aman and Piotrowski, 2008; Haas and Gilmour, 2006). This is similar to PGCs where lack of *cxcl12a* does not impair protrusion formation and

tumbling motility (Blaser et al., 2005). Although numerous mutations and manipulations cause primordium stalling, none of these have been able to abolish motility (tumbling) of individual cells within the tissue. It remains to be tested whether, in analogy to the roles of JAK/STAT and PCP signaling in *Drosophila* border cells, the STAT or PCP pathway are required for the onset and maintenance of motility of the posterior lateral line primordium.

Similar to PGCs, Cxcr4b-Cxcl12a chemokine signaling is the major chemoattractive system in the posterior lateral line primordium (David et al., 2002; Li et al., 2004). *cxcl12a* is expressed in cells along the horizontal myoseptum prefiguring the track on which the posterior lateral line primordium migrates (Figure 4.3D; blue stripe). Cxcl12a is necessary for directing cell protrusions toward the tail of the embryo, as loss of *cxcl12a* leads to non-directed, random protrusion formation (Haas and Gilmour, 2006). In contrast to border cell migration, the gradient of chemoattractant is not due to passive diffusion from a source of ligand at the migration target. Rather, *cxcl12a* mRNA is present in a uniform stripe along the prospective migratory path (David et al., 2002). Although a Cxcl12a protein gradient spanning the AP axis has not been ruled out, genetic experiments show that the primordium is capable of migrating in either direction along the uniform stripe of *cxcl12a* mRNA. For example, in N-cadherin mutants, in which somites and the horizontal myoseptum are partially disrupted, the primordium occasionally performs a U-turn (Kerstetter et al., 2004).

Two receptors for Cxcl12a are expressed in the primordium. *cxcr4b* is expressed broadly in the leading portion of the primordium and *cxcr7b* is only

expressed in about the trailing one third of the tissue (Figure 4.3D; Dambly-Chaudiere et al., 2007; David et al., 2002; Valentin et al., 2007). In the absence of a simple, diffusion based Cxcl12a gradient, this primordium polarization itself is likely the key mechanism allowing directional migration (Dambly-Chaudiere et al., 2007, Valentin et al., 2007). Chemokine receptor asymmetry is crucial for directional migration, as loss of either receptor leads to stalling of the tissue. Similarly to what has been observed in chemoattractant deficient PGCs and border cells, loss of directional migration does not lead to a loss of cell motility but cells migrate along random independent vectors effectively abolishing correct directional migration (Aman and Piotrowski, 2008; Dambly-Chaudiere et al., 2007; Doitsidou et al., 2002; Haas and Gilmour, 2006; Prasad and Montell, 2007). In analogy to PGCs, an attractive hypothesis is that that Cxcr7b receptors expressed in trailing cells of the primordium function as a Cxcl12a sink to reduce the concentration of Cxcl12a available for Cxcr4b binding. Cxcl12a sequestration by Cxcr7b possibly leads to the formation of a dynamic Cxcl12a protein gradient across the primordium enabling directional migration on a uniform stripe of Cxcl12a (Aman and piotrowski, 2009; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). This mechanism also explains why in certain experimental contexts the primordium can turn and migrate in the opposite direction along the *cxcl12a* stripe. As long as primordium polarity is maintained, a dynamic gradient of Cxcl12a protein can be produced by the migrating primordium itself.

The significance of primordium polarity for directed migration raises the question of what mechanisms initiate and maintain this polarity. Primordium

polarization, and thus chemokine receptor asymmetry, is maintained by a paracrine feedback mechanism involving asymmetric Wnt/β-catenin and Fgf pathway activation (Figure 4.3E; Aman and Piotrowski, 2008). Activation of Wnt/β-catenin signaling in cells occupying the leading portion of the cluster leads to expression of secreted Fgf ligands. However, Wnt/β-catenin pathway activation simultaneously upregulates the membrane-bound Fgf pathway inhibitor *sef* preventing Fgf pathway activation in leading cells. As Fgf ligands are free to diffuse out of this inhibitory domain, they stimulate expression of target genes in the trailing portion of the tissue. Fgf signaling, in turn, restricts Wnt/β-catenin target genes to the leading zone by inducing *dkk1* expression in trailing cells. Wnt/β-catenin inhibits *cxcr7b* in leading cells and promotes *cxcr4b* expression by inhibiting an unidentified repressor of *cxcr4b*. Thus, the reciprocal interactions between Wnt/β-catenin and Fgf signaling are critical to maintain polarized expression of the chemokine receptors *cxcr4b* and *cxcr7b* and for sustained directional collective migration (Figure 4.3E).

Even though we understand how primordium polarity is maintained, it remains enigmatic how primordium polarity is initially established after placode induction. Chemokine signaling polarizes cells in multiple systems, making it an attractive candidate signaling pathway possibly involved in polarizing the lateral line primordium. However, primordium polarity forms normally in the absence of Cxcl12a (Aman and Piotrowski, 2008). Also, the Wnt ligand has not been identified yet. Thus, the Wnt signal could be provided by the environment or it could be produced by cells at the leading edge. In this second scenario

primordium polarity is maintained and reinforced by signaling interactions between the cells of the cluster without requiring input from the underlying tissues.

A mechanism by which collectively migrating cells express different genes in the leading and trailing regions could have implications for collective cancer invasion. Small differences in gene expression among tumor cells might be reinforced by paracrine feedback loops leading to cluster polarization and onset of migration and invasion. Also, if cluster polarity indeed maintains itself in the absence of signals from surrounding tissues, this could explain why groups of cancer cells are able to migrate through very diverse tissues. Interestingly, the leading cells of invasive cancer collectives express high levels of matrix remodeling enzymes, including Mmp14, which are targets of Wnt/β-catenin signaling in colorectal adenoma (Benini et al., 2005). The role of Wnt/β-catenin signaling in polarizing collectively migrating invasive tumors has not been evaluated.

It is still enigmatic how the posterior lateral line primordium stops migration at the tail tip. It is possible that, similar to stalling PGCs and border cells, the primordium encounters a region of uniform chemoattractant in this region. Indeed, *cxcl12a* mRNA is expressed much more broadly in the tail tip than along the horizontal myoseptum (Fig 4.3D). However, it is unclear whether this mechanism is sufficient to stop the primordium as *cxcr7b* expression in cells at the rear of the primordium generates a dynamic Cxcl12a gradient. It is also possible that upon reaching the tail tip, chemokine independent signals cause the

primordium to lose protrusions and differentiate as neuromasts. Live imaging and gene expression analysis of primordia as they reach the tail tip and cease forward migration is required to shed light on this question.

Angiogenic sprouting: collective migration of thin cellular filaments

Angiogenic sprouting in vertebrates is defined as the formation of new blood vessels from existing vessels. During embryonic and postnatal development, networks of blood vessels undergo significant remodeling and elaboration in order to completely perfuse tissues ensuring an adequate blood supply. Angiogenic sprouting is one of the major mechanisms used to remodel and elaborate vessel networks. This review will briefly focus on regulation of sprout collective migration in the postnatal mouse retina to allow comparisons with other models of migration (Figure 4.4A, A'). Angiogenic sprouts are composed of a single tip cell followed by a variable number of stalk cells. As these cells remain tightly adhered to each other during migration, this process is considered an example of collective cell migration. However, it is distinct from the collective cell migration of the border cell cluster and the posterior lateral line primordium. The sprout remains attached to the parent vessel and is generally a thin filament of cells rather than a cluster of cells (Figure 4.4A').

The mouse retina has proven to be a potent model for elucidating mechanism of endothelial cell migration during angiogenesis. Angiogenesis occurs in the mouse retina after birth and involves the formation of an elaborate vascular network from a simple capillary ring formed at the center of the

embryonic retina. Sprouts emerge from these vessels and migrate toward the periphery of the retina, branching and proliferating as they go leading to the formation of a complicated network of highly branched vessels (Figure 4.4A; for comprehensive reviews of the mouse retina angiogenesis model see Fruttiger, 2007; Gerhardt, 2008; Uemura et al., 2006).

The first step in angiogenic sprouting is the specification of a highly motile tip cell from among quiescent endothelial cells of an existing blood vessel. In the mouse retina VEGF-A is necessary and sufficient for the specification of tip cells (Gerhardt et al., 2003). The tip cell upregulates Delta ligands, such as Dll4 in retinal sprouts, which subsequently signal through Notch receptors present throughout the quiescent vessel to limit the acquisition of tip cell fate to a few cells. Migrating cells in the rest of the sprout specified by the action of Delta-Notch signaling from the tip are known as stalk cells (Claxton and Fruttiger, 2004; Hellström et al., 2007; Krebs et al., 2000). Stalk cells do not form elaborate protrusions like tip cells and may not actively contribute force for motility to the elongating sprout (Fig 4.4A'; Gerhardt et al., 2003).

Once specified by VEGF-A, tip cells orient and migrate from the central retina toward the peripheral retina which expresses higher levels of VEGF-A, causing radial growth of the vascular plexus (Figure 4.4A). As the retina matures, VEGF-A production is stimulated by local hypoxia ensuring that vascular sprouts grow into regions that require increased vascular coverage (Stone et al., 1995). The result of this process is a highly branched network of blood vessels that entirely perfuses the retina. In contrast to the examples discussed above, onset

of motility and directed migration are regulated by the same signaling molecule, VEGF-A. This ligand is capable of both stimulating protrusions in nascent tip cells and orienting these protrusions toward their targets. A unique feature of angiogenic sprouts is that they stop migrating because they induce downregulation of their chemoattractant VEGF-A. As sprouts migrate into regions of local hypoxia and begin delivering blood to these tissues, hypoxia is relieved and VEGF-A expression subsides (Stone et al., 1995). Therefore, in contrast to border cells and PGCs, which stop migrating upon reaching a region of uniform chemoattractant, angiogenic sprouts stop migrating due to a loss of protrusions caused by a downregulation of chemoattractant.

Interestingly, the VEGF-A isoforms produced in the retina bind tightly to heparin components of the ECM secreted by retinal astrocytes, which forms a functional VEGF-A protein gradient. Therefore, overexpressing VEGF-A or expressing a non-heparin binding isoform of VEGF-A destroys the gradient and causes impaired sprout migration (Gerhardt et al., 2003). Thus, a general feature of *in vivo* cell migration is the requirement for post-translational regulation of chemoattractant ligands to generate gradients in extracellular space.

Neural crest migration: collective migration of cellular streams

Neural crest cells have been a classical model to study cell migration *in vivo* (Le Douarin, 2004). Neural crest cells arise along the border between neural and non-neural ectoderm. These cells subsequently delaminate from the dorsal neural tube and migrate throughout the embryo to give rise to neural, as well as non-neural tissues (Figure 4.4B; Knecht and Bronner-Fraser, 2002). Once they

reach their respective targets, neural crest cells differentiate into cartilage, pigment cells, sensory neurons, and ganglia and contribute cells to the sympatho-adrenal glands. At a gross morphological level neural crest cells appear to migrate in loosely associated chains. However, scanning electron micrographs and live imaging have demonstrated that cells communicate via fillipodia and that cell-cell communication is crucial for directed migration (Fig 4.4B'; Bancroft and Bellairs, 1976; Davis and Trinkhaus, 1981; Teddy and Kulesa, 2004).

Neural crest cells form in dorso-lateral regions of the neural tube and, as they begin to migrate, undergo an epithelial to mesenchymal transition (EMT). EMT is triggered by several signaling pathways, chiefly BMP, FGF and WNT (reviewed in Acloque, 2009). Downstream of these signaling pathways, transcription factors such as Snail and Foxd3 that modulate cell-cell adhesion and cell polarity are activated and thus enable cells to leave the neural epithelium. For example, the zinc-finger transcription factor Snail represses E-cadherin, which in turn is crucial for modulating adherens junctions (Nieto, 2002). Snail also acts as a repressor of genes regulating tight junction proteins or proteins involved in the establishment of apico-basal polarity (reviewed in Acloque, 2009; Ikenouchi et al., 2003; Peinado et al., 2007). Interestingly, a recent study demonstrated that the cell-adhesion molecule Cadherin-11 not only affects neural crest cell adhesion but also directly promotes migration (Kashef et al., 2009). Cadherin-11 regulates fillipodia and lamellipodia formation via guanine nucleotide exchange factor (GEF)-Trio and the small Rho GTPases (Jaffe and

Hall, 2005). Thus, cell adhesion molecules play multiple important roles in the regulation of migration, which have to be tested in other model systems.

So far, all well-described guidance molecules involved in neural crest cell migration are repulsive in nature. Among these are the ligand receptor pairs Robo/Slit, Neuropilin/Semaphorin and Ephrins/Eph (reviewed in Kuriyama and Mayor, 2008). Especially, the noncanonical Wnt/planar cell polarity (PCP) pathway is essential for directional migration of neural crest cells. The PCP pathway stabilizes protrusions and in its absence, protrusions form in a non-directed fashion (De Calisto et al., 2005). Importantly, the PCP pathway is responsible for contact inhibition. As cells touch each other, the PCP pathway is locally activated at zones of contact leading to activation of RhoA and collapse of cell protrusions (Carmona-Fontaine et al., 2008). Thus, leading cells are repelled by follower cells causing efficient directed migration. However, even though contact inhibition surely plays an important role, it does not exclude the possibility that attractive chemokine signaling is also involved in guiding neural crest migration. In support of an involvement of chemotaxis Cxcl12 promotes migration of cultured neural crest-derived dorsal root ganglion cells and *cxcr4a* and *cxcl12a* are expressed in the zebrafish pharyngeal arches, possibly guiding cranial neural crest cells (Belmadani et al., 2005; Thisse, 2001).

Neural crest cells stop migrating in areas where repulsive signals are low. For example, trunk neural crest cells migrate away from the neural tube to form sympathetic ganglia ventral of the somites. Cranial neural crest cells coalesce

into ganglia by integrating repulsive signals such as Ephrin/Eph in interganglionic regions and attractive cues, such as N-cadherin (Kasemeier-Kulesa et al., 2006).

Vertebrate gastrulation

Gastrulation movements are driven by several mechanisms such as polarized planar and radial intercalations, cell shape changes, and active cell migration (Figure 4.4C; Keller, 2005; Rohde and Heisenberg, 2007; Solnica-Krezel, 2005). For instance, *Xenopus* anterior mesendoderm actively migrates (Figure 4.4D), whereas trunk mesodermal cells intercalate (not shown). Both processes are regulated by different molecular mechanisms exemplified by the fact that they respond differently to the activation of Rho-GTPases (Ren et al., 2006). Additionally, cells in different germ layers may utilize distinct migration strategies at the same developmental stage. For instance, early in zebrafish gastrulation mesodermal cells directionally migrate toward the animal pole while endodermal cells spread toward the animal pole by an active, nondirected random walk (Pézeron et al., 2008). The multitude of movements that govern gastrulation makes it difficult to define the steps characteristic of other models of cell migration (Figure 4.1). Nevertheless, we would like to briefly summarize findings that have been made by studying gastrulation movements that have relevance to our understanding of cell migration in general.

Before mesodermal cells commence active migration they undergo EMT similar to neural crest cells. Studies in mice demonstrated that, during gastrulation, EMT is induced by Fgf which upregulates Snail. Snail, in turn, downregulates E-cadherin causing cells to acquire a mesenchymal character

(Carver et al., 2001; Ciruna and Rossant, 2001). Studies in zebrafish identified an additional pathway that induces EMT via the activation of the transcription factor Stat3 and its downstream target LIV1 (Solnica-Krezel, 2005; Yamashita et al., 2002; 2004).

Similarly to neural crest cells, migrating dorsal mesodermal cells are of mesenchymal character that are loosely connected and extend many protrusions toward the direction of migration (Lawson and Schoenwolf, 2001; Ulrich et al., 2003; Winklbauer et al., 1996). Although they are only loosely connected, cells migrate as a coherent sheet that optimizes cell migration. Explant experiments have demonstrated that individual cells migrate more slowly than when they are part of a sheet of cells (Davidson et al., 2002; Ren et al., 2006). Similarly to posterior lateral line primordium migration, more posterior cells extend cell protrusions underneath the preceding cells (Winklbauer and Nagel, 1991; Winklbauer and Selchow, 1992). In contrast, zebrafish endoderm cells initially migrate as isolated individuals with no interactions between the migrating cells (Pézeron et al., 2008).

In *Xenopus*, anterior mesendoderm cells are guided toward the blastocoel roof by the growth factor PDGFA (Figure 4.4D). Interestingly PDGFA is not required for mesendoderm protrusions and motility. Therefore, loss of PDGFA results in randomized migration of mesendoderm cells (Nagel et al., 2004). This is similar to the role of chemoattractant guidance molecules in PGCs, border cells, and the lateral line primordium. Additionally, during *Xenopus* gastrulation, *cxcl12a* is expressed in cells of the blastocoel roof and *cxcr4* is expressed in the

leading edge of the migrating anterior mesendoderm (Figure 4.4D; Fukui et al., 2007). Overexpression or knockdown of *cxcl12a* severely impairs migration demonstrating a role for chemokine signaling in mesendoderm migration. Explant experiments show that mesendoderm cells migrate toward blastocoel roof cells *in vitro* and that this migration requires chemokine signaling, as no migration occurs unless the blastocoel roof explants express *cxcl12a* and the mesendoderm explants expresses *cxcr4* (Fukui et al., 2007).

In zebrafish, early endoderm migration toward the animal pole occurs via a undirected random walk that serves to evenly populate the inner surface of the blastoderm with cells, whereas later migration toward the dorsal side of the embryo relies on directional migration (Figure 4.4C, black arrows; Pézeron et al., 2008). Two studies revealed that chemokine signaling is essential for the later dorsal migration of endoderm (Mizoguchi et al., 2008; Nair and Schilling, 2008). *cxcr4a* is expressed in the endoderm, whereas the ligands *cxcl12b* and *cxcl12a* are expressed in the mesoderm on top of which the endoderm migrates. In the absence of *cxcr4a* or *cxcl12a,* the anterior endoderm is displaced and has defects in its dorsal migration. However, ectoderm and mesoderm migration is normal. The two groups came to different conclusions with regard to the underlying molecular mechanisms of the endoderm migration defect. Mizoguchi et al. concluded that chemokine signaling is important for guiding endodermal cells, whereas Nair and Schilling's data imply that chemokine signaling is crucial for Integrin mediated adhesion (discussed below).

In zebrafish, *cxcr4a* is expressed in the endoderm, whereas the ligands *cxcl12b* and *cxcl12a* are expressed in the mesoderm on top of which the endoderm migrates. Mizoguchi et al. characterized the phenotype of *cxcl12a/b* morphant embryos as a loss of chemotaxis as live imaging revealed that endodermal cells extend fewer protrusions and that these protrusions are not properly oriented along the direction of migration (Mizoguchi et al., 2008). Furthermore, in the absence of chemokine signaling, endodermal cells were observed to migrate with similar speed as endodermal cells in control embryos but their directionality was impaired. These data were interpreted to show that mesodermally expressed Cxcl12a/b is acting as a chemoattractant to guide the Cxcr4a expressing endoderm. In support of this conclusion, endodermal cells cluster around ectopic patches of *cxcl12a/b* in *cxcl12a/b* MO embryos (Mizoguchi et al., 2008).

Over the past few years it has become increasingly clear that, in addition to guidance cues, cell adhesion molecules play an essential role in regulating gastrulation movements (Hammerschmidt and Wedlich, 2008; Solnica-Krezel, 2006; Witzel et al., 2006). Epiboly movement and prechordal mesoderm migration depend on E-cadherin (Kane et al., 2005; Montero et al., 2005). Similarly to what has been described for Cadherin-11 function during neural crest migration, Fibronectin-Integrin interactions are not only essential for cell adhesion but also for lamellipodia formation (Hammerschmidt and Wedlich, 2008; Winklbauer and Keller, 1996), development of directed protrusions (Davidson et al., 2006), and cell polarity (Marsden and DeSimone, 2001).

Interestingly, chemokine signaling has also been demonstrated to control ECM-integrin-dependent adhesive interactions between the endoderm and the mesoderm by regulating *integrin* transcription in the endoderm (Nair and Schilling, 2008). This conclusion is supported by the finding that zebrafish *cxcr4a*-depleted cells adhere much less efficiently to Fibronectin-coated substrates and that the migration defect observed in *cxcr4a* morphant embryos can be rescued by injection of *integrin* (*itgb1b)* mRNA (Nair and Schilling, 2008). It is possible that the clustering of endoderm cells around ectopic *cxcl12a* observed by Mizoguchi et al. is also due to the regulation of integrin mediated adhesion rather than chemotaxis. In this interpretation, endoderm cells stop migrating on ectopic patches of *cxcl12a* expression due to strong adhesion to the Fibronectin-containing ECM that overlies them. Likewise, the defects in protrusion formation described by Mizoguchi et al. could be due to loss if integrin mediated adhesion. It remains an interesting challenge to elucidate how chemokine signaling mediated adhesion is coordinated with guidance to ensure correct migration. Moreover, it will be crucial to determine whether the integrin mediated adhesion mechanism operates in other examples of chemokine guidance such as migration of PGCs or the lateral line primordium.

Regulation of morphogenic migration

As we have seen, live imaging combined with genetic analyses has yielded a wealth of new information about how cell migration is regulated *in vivo*. A general theme that has emerged is that cell migration, in many cases, is regulated at three different steps. Prior to migration, cells have a simple

morphology and lack protrusions (Figure 4.1A). In the first step, cells elaborate protrusions in all directions (Figure 4.1B). In the second step, protrusions are oriented in the direction of migration and the cells move (Figure 4.1C). Finally, in the third step cells cease moving upon reaching their destination (Figure 4.1D). Although this concept was originally developed through the study of PGC migration (Blaser et al., 2005), it appears that it is generally applicable to most examples of embryonic cell migration. Below we highlight similarities as well as differences between the models discussed above (also see Table 4.1).

Phase I: acquisition of a complex cell morphology
and protrusion formation

To begin migrating, a cell must gain competence to respond to directional cues and, in the case of cells of epithelial origin, detach from neighboring, non-motile cells (Figure 4.1A). Although these processes are among the most clinically relevant aspects of morphogenic cell migration, they appear to be among the least well understood. In general, these processes involve down regulation of specific adhesion molecules involved in tissue integrity and up regulation or spatial segregation of components that regulate the cytoskeleton and generate dynamic traction forming adhesions. During this premigratory phase cells acquire a more complex morphology and begin to extend cell protrusions, such as thin fillipodia for guidance and larger lamellipodia for traction generation in a nondirected fashion.

One surprising conclusion from the study of developmental cell migration is that the molecular mechanisms that regulate the acquisition of motility are

often distinct from the mechanisms that regulate later directional migration. For example, PGCs and lateral line primordium cells become motile in the absence of chemokine signaling molecules and border cells become motile in the absence of growth factor chemoattractants (Aman and Piotrowski, 2008; Haas and Gilmour, 2006; Valentin et al., 2007) . Likewise, in zebrafish PGCs, overexpression of the chemokine guidance molecule *cxcl12a* does not lead to activation of intracellular chemokine signaling and directional cell migration until after PGCs have formed protrusions (Blaser et al., 2005). The onset of motility in gastrulation movements and neural crest migration also appear to be regulated by signaling pathways that are distinct from those necessary for guidance. The acquisition of protrusions in these cells is regulated by pathways that control EMT rather than guidance.

These *in vivo* studies contradict conclusions drawn from *in vitro* studies, which put forward the attractive concept that chemoattractant gradients induce polarized cellular extensions on the side of the cell facing the higher concentration of chemoattractant (reviewed in (Burridge and Wennerberg, 2004)). Interestingly, independent regulation of protrusion acquisition and guided migration occurs in cells that migrate as isolated individuals, as well as cells migrating as multicellular collectives.

It is not known whether the regulation of protrusion formation is controlled by similar molecules across model systems. Regulation of this phase of migration is by far best understood in *Drosophila* border cells, where the coordinated activity of JAK/STAT, Notch and PCP signaling is necessary for the correct

formation of protrusions (Bastock and Strutt, 2007; Beccari et al., 2002; Prasad and Montell, 2007). It remains to be investigated whether pathways uncovered in border cells also regulate this process in these other systems and therefore represent potentially conserved functional regulatory mechanisms. In PGCs, a micro-RNA binding molecule called ‘Dead end’ is necessary for the acquisition of motility but the down-stream mechanism is not understood (Weidinger et al., 2003). To date, no experimental manipulation has been able to abolish motile behavior from cells of the lateral line primordium.

In contrast to the model systems described above, acquisition of endothelial cell protrusions, as well as control of subsequent directional migration during formation of angiogenic sprouts in the mouse retina requires the same molecule, VEGF-A (Gerhardt et al., 2003). Therefore, while utilizing distinct regulatory mechanisms for the acquisition of motility and later directional migration may be a widespread phenomenon, it is not present in all migrating cells. Live imaging analysis of cell in which migration is blocked by loss of guidance information is necessary to evaluate whether a given cell fails to migrate due to failure to acquire protrusions or loss of guidance.

Phase II: polarization, detachment and directional migration

Once cells have gained the ability to generate protrusions and traction forces they become polarized and point their protrusions in the direction of migration. This process coincides with the onset of directional migration (Figure 4.1B). Common to migrating cells is that they are guided via chemoattractant ligands, most commonly chemokines or growth factors, as in the examples

discussed here. In neural crest cells repellant molecules also play an important role. The direction of migration is informed by gradients of these attractant and repellant molecules in the environment. Differences exist on how these gradients are generated and how the ensuing signals are interpreted intracellularly by the migrating cells leading to polarization of membrane protrusions and directional migration.

A chemoattractant gradient can be established via several mechanisms. The simplest mechanism to establish a chemoattractant gradient is free diffusion of ligand from the target tissue. For example in *Drosophila* border cell migration growth factor chemoattractants are produced in the oocyte from where they diffuse to the anterior pole of the egg chamber (McDonald et al., 2003; 2006). A second mechanism involves the post-translational regulation of guidance ligands in extracellular space. A striking example is the formation of the Cxcl12 gradient via interactions with the newly described Cxcr7b receptor. Binding of Cxcl12a to Cxcr7b does not activate an intracellular signaling cascade, but rather leads to the internalization and destruction of Cxcl12a (Boldajipour et al., 2008). Thus, in PGCs, and likely in the lateral line primordium, Cxcr7b is involved in limiting the concentration of extracellular Cxcl12a chemoattractant in a spatially restricted manor. Therefore, loss of Cxcr7b leads to the failure of establishing a chemokine gradient and loss of directional migration.

Retinal vascular sprouts provide another example where post-translational regulation of guidance cues is important for correct gradient formation and directional migration. In this case the chemoattractant VEGF-A must associate

with heparin present on neighboring astrocytes for efficient gradient formation (Gerhardt et al., 2003). Heparin likely plays a similar role in the regulation of gastrulation movements (Itoh and Sokol, 1994).

Similar mechanisms might be used to generate chemoattractant gradients as are employed in generating gradients of patterning morphogens. Recent studies of Fgf8 behavior suggest that free diffusion of signaling molecule coupled with receptor mediated endocytosis is sufficient to generate a stable gradient (Yu et al., 2009). Such a 'source-sink' model also operates in establishing Cxcl12a gradients that guide PGCs and the lateral line primordium toward their destinations, as described above.

Phase III: termination of migration

The final regulatory step of morphogenic cell movements is termination of migration when the cells reach their target sites (Figure 4.1D). In general, this appears to be a relatively poorly understood aspect of cell migration. In cases where the molecular signals that stop migration have been elucidated, cells cease to migrate as they reach a region of locally high attractant or are surrounded by repulsive cues. For example, the highest concentration of growth factors is present close to the *Drosophila* oocyte in the egg chamber where border cells will contribute to the formation of the sperm entry site (McDonald et al., 2006; McDonald et al., 2003). Similarly, *cxcl12a* is highly expressed at the prospective gonad where zebrafish PGCs stop migrating (Doitsidou et al., 2002). In these cases, cells orient along a chemoattractant gradient and will not be able to leave a region of uniform or locally high guidance molecule concentration.

A second possible mechanism for the termination of migration could rely on physical impedance based on the morphology of the target tissue. For example, in the case of the posterior lateral line primordium and *Drosophila* border cells one might envision that the tail tip and the oocyte present physical barriers, respectively. In the egg chamber the oocyte is in direct contact with follicle cells on all sides. During migration, the border cell cluster is able to migrate between nurse cells, but once it reaches the oocyte, further migration would require cell invasion. However, in zebrafish PGCs the effect of a physical barrier and high levels of signaling can be dissociated. Aberrant chemokine signaling causes PGCs to overshoot past the gonads, and ectopic expression of *cxcl12a* can cause PGCs to stall in islands of highly *cxcl12a* expressing cells. These findings demonstrate that termination of PGC migration is not dependent on the presence of a physical barrier (Boldajipour et al., 2008; Reichman-Fried et al., 2004).

A third possibility is that other signaling interactions at these destinations lead to a loss of protrusions such that the cells are no longer able to respond to attractive cues altogether. As cells reach their target and differentiate, they could become nonmotile and form functional components of mature organ systems. The *in vivo* factors that turn off motility have not been uncovered in any system but will likely involve the downregulation of factors that contribute to the initial acquisition of protrusions such as JAK/STAT signaling in border cells. This might make it difficult to evaluate this stopping mechanism *in vivo*, as loss of function in genes necessary for motility will result in impaired initiation of migration.

Finally, a fourth stopping mechanism is exemplified by angiogenic sprouts in the retina. As migrating sprouts reach their target locations they form new vessels permitting blood flow. Subsequently, the tissue is oxygenated, which relieves hypoxia. Hypoxia dependent VEGF-A expression is downregulated and the sprout tip loses protrusions (Gerhardt et al., 2003; Stone et al., 1995). Therefore, angiogenic sprouts trigger the downregulation of chemoattractant upon reaching their destination.

Resolution of these possibilities in a given system will require live imaging of clusters at the end of migration and conditional disruption of gene function. If cells at the end of the migratory pathway are still motile, extend protrusions and tumble, a uniform level of attractant is likely causing cessation of migration. On the other hand, if cells lose motility altogether as they reach the target, it is more likely that other signals from the environment shut off cell motility. Elucidating such signals could have great clinical importance, as their inhibition might impede or prevent cancer dissemination by blocking the acquisition of motility. Although not as well studied as the other aspects of morphogenic collective migration, termination of migration is a vital step during morphogenesis with tremendous potential clinical interest that deserves more study in the future.

Additional considerations

Individual versus collective cell migration

A major difference between individually and collectively migrating cells is how they interact with their environment and how they sense/process directional cues. Although zebrafish PGCs and lateral line primordia rely on the same set of genes for guided migration, interesting differences between these systems spring from the fact that PGCs migrate as individual cells and the lateral line primordium migrates as a multicellular collective. During PGC migration Cxcr7b (a Cxcl12a sink) is dynamically expressed in nonmigrating cells surrounding the migrating PGCs to sharpen a broad Cxcl12a gradient (Boldajipour et al., 2008). The lateral line primordium, however, expresses *cxcr7b* in migrating cells themselves enabling the primordium to migrate along a presumably uniform path of Cxcl12a (Aman and Piotrowski, 2008; Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Thereby, directional information is created by signaling interaction within the migrating collective itself, as well as signals from the environment. Similarly, communication between cells in other migrating collectives such as border cells, neural crest cells and vascular sprouts are also vital for normal directional migration.

Another important difference is that collectively migrating cells modify the surrounding extracellular matrix (ECM), whereas individual cells squeeze through the ECM in an amoeboid fashion (Friedl and Gilmour, 2009; Friedl and Wolf, 2003). This finding by itself suggests that single cells should be able to migrate faster. However, in the systems thus far investigated, collective cell migration

appears more efficient than single cell migration. For example isolated *Xenopus* mesodermal cells have difficulty migrating directionally along the blastocoel roof (Winklbauer et al., 1992). Also, neural crest cells in which cell-cell adhesion is compromised by disrupting N-cadherin migrate more slowly. Likewise, it has been reported that individual neural crest cells do not migrate well in vivo, likely because of a lack contact inhibition (Patrick Pla et al., 2001). One explanation for this phenomenon is that cells connected via cell-cell adhesion respond to forces coming from neighboring cells, whereas single cells solely rely on cell-substrate interactions. Also, groups of cells generate more force, as measured by the number of total focal adhesion points and traction forces (du Roure et al., 2005; Kolega J, 1982). Another possible explanation for the efficiency of collective cell migration is that collectively migrating cells are more proficient in interpreting guidance signals. In general, a migrating collective spans more area than a single cell and can therefore potentially detect shallower gradients of guidance molecules. In addition, cell-cell communication in a migrating collective aids in directed migration. In collectively migrating cells only a few tip cells must perceive guidance cues (Haas and Gilmour, 2006), whereas individual cells continuously sample the environment to detect a gradient of an attractant. For example, individually migrating PGCs exhibit tumbling phases during which they do not move but explore the environment. The tumbling phase itself occurs cell-autonomously and independently of chemokine signaling and it was suggested that it might serve to redirect the cells (Reichman-Fried et al., 2004). Such tumbling phases are not observed in migrating collectives.

As single cell and collective cell migration occur simultaneously in an organism, the question arises whether cells are locked into their particular mode of migration. Surprisingly, studies of cancer cells revealed that migratory cells exhibit a large degree of plasticity. Collectively migrating cancer cells proteolytically degrade the extracellular matrix during forward migration. Disruption of their ability to remodel the surrounding ECM with pharmacological inhibitors of proteases was expected to yield groups of cells ‘stuck’ in the tissue. However, instead, these cancer cells switched their migratory mode from collective cell migration to a mesenchymal or amoeboid migration (Friedl and Wolf, 2003; Wolf et al., 2003).

An interesting question is whether collectively migrating cells during normal development are also able to migrate as individual cells if challenged. Manipulations of neural crest stream and border cell cluster integrity provide us with some answers. At a gross morphological level neural crest cells appear to migrate in loosely associated chains. However, scanning electron micrographs and live imaging have demonstrated that cells communicate via fillipodia and that cell-cell communication is crucial for directed migration (Bancroft and Bellairs, 1976; Davis and Trinkhaus, 1981; Teddy and Kulesa, 2004). For example, in transgenic mice with disrupted gap junction communication, cardiac neural crest cells migrate aberrantly (Sullivan et al., 1998). Gap junctions localized in cell membranes allow the passage of second messengers, ions and small metabolites, and thus could aid in transmitting guidance signals from leader cells to followers (Roberto Bruzzone et al., 1996).

A similar effect has been seen in *Drosophila* border cells in which the *hindsight* gene (*hnt*) is disrupted (Melani et al., 2008). *Hnt* is a negative regulator of JNK. JNK is essential for maintenance of cell polarity and cell-cell contacts. In its absence the border cell cluster disintegrates (Llense and MartÌn-Blanco, 2008). Nevertheless, individual border cells still migrate slowly, as long as they maintain contact with the polar cells while extending multiple protrusions in all directions. Thus, motility of groups of cells, such as neural crest and border cells does not absolutely depend on cohesiveness of the migrating cells, however, when isolated, these cells fail to undergo proper morphogenesis. It has not been determined yet if this failure is caused by an inability to efficiently integrate guidance cues, or whether their slowed migration causes them to reach their targets too late, at which point signals from the environment have changed. Likely, as cancer cells do not follow a precise developmental program and form morphological structures that have to be integrated into the organ system, they are more flexible with respect to their migration mode. During development however, changes in the mode of migration appear detrimental for morphogenesis.

Epithelial polarity and migration

Neural crest and many cancer cells undergo an EMT as they begin to migrate (Thiery, 2003). For these cell types it is essential to lose their polarity, so they can emigrate from the neural tube or away from a tumor. However, border cells remain apico-basally polarized during migration with their apical domains facing away from the polar cells. This configuration is established by the action of

the Par/aPKC polarity complex (McDonald et al., 2008). Lateral line primordium cells are also apico-basally polarized while migrating, which also requires the action of classical apico-basal determinants such as aPKC. aPKC localizes zonula adherens junctions to the distal side of cells (the side facing away from the somites) likely by regulating the localization of Par proteins (Hava et al., 2009). In the trailing two-thirds of the primordium apico-basally polarized cells constrict apically leading to the formation of rosette shaped proneuromasts (Lecaudey et al., 2008). In addition, sensory hair cells in deposited neuromasts are also polarized with cilia either oriented in parallel or perpendicular to the antero-posterior axis (Lopez-Schier and Hudspeth, 2006). However, it has not yet been determined whether hair cell polarity is established in the precursors during migration or only once proneuromasts are deposited.

Even though apical-basal cell polarity is essential for the initiation of border cell migration, it is likely not required for lateral line primordium migration as has been previously suggested (Lecaudey et al., 2008). Inactivation of Wnt/ß-catenin signaling in the cluster leads to the loss of Fgf signaling (Aman and Piotrowski, 2008). Loss of Fgf signaling, in turn, is accompanied by a loss of apico-basal polarity and rosette formation (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Yet, in the absence of Wnt/β-catenin signaling primordia still migrate to the tip of the tail, even though they fail to form and deposit proneuromasts (Aman and Piotrowski, 2008).

In contrast to apico-basal polarity, polarity of cellular protrusions is indispensable for directed migration. All migrating cells elaborate cellular

extensions that presumably help generate the traction forces necessary for directional migration. As mentioned above, protrusion polarity is controlled by gradients of chemoattractant signaling molecules. For example, in the absence of Cxcl12a, PGCs and lateral line primordium cells form protrusions in random positions, cells begin to tumble and migration stalls. Thus, chemoattractant induced cell polarity is essential for directed cell migration in all well studied model systems, whereas apico-basal polarity is only required in a few.

Induction of motility as a mechanism to activate dormant cancer stem cells?

JAK/STAT signaling has been recognized as an important target in cancer therapy, as JAK/STAT signaling is essential for the onset of migration, directed cell migration and homing of many cancer cell types to particular organs where they form metastases (Liang et al., 2004). Signals that activate JAK/STAT signaling during these migratory events are, for example, chemokines and cytokines, such as Cxcl12 and interferons (Essers et al., 2009; Vila-Coro et al., 1999). Inhibiting the onset of migration is a powerful approach to inhibit cancer cell dissemination. However, in other instances, such as leukemia stem cells, it might be advantageous to promote cell motility. Dormant leukemia stem cells divide very rarely and are therefore not susceptible to antiproliferative drugs (Essers et al., 2009; Goldman and Gordon, 2006; Lerner C, 1990). The persistence of dormant stem cells in the bone marrow causes a high remission rate among cancer patients after treatment. Recent elegant work by Essers at al. 2009 has shown that dormant hematopoietic stem cells (HSCs) can be activated

by interferon alpha (INFα), a cytokine produced during an inflammatory response or during infections. Treated, activated HSCs begin to proliferate and are efficiently targeted and depleted by chemotherapeutic agents. INFα mediates its effects via Jak/Stat signaling and it is thought that activation of JAK/STAT leads to proliferation of stem cells (Briscoe et al., 1996; Darnell JE Jr, 1994; Essers et al., 2009; van Boxel-Dezaire et al., 2006). However, as onset of proliferation normally coincides with stem cells leaving their niche (Wilson and Trumpp, 2006), an attractive hypothesis is that activation of JAK/STAT primarily causes the onset of motility of dormant stem cells. As described above, chemokines also signal via JAK/STAT(Vila-Coro et al., 1999). Chemokine signaling has been shown to be essential for homing and mobilization of neutrophils. In the presence of high levels of the chemokine ligand CXCL12 neutrophils leave their niche in the bone marrow (Furze and Rankin, 2008). These observations raise the possibility that IFNα might activate/mobilize stem cells via the induction of chemokine receptors. This hypothesis is conceivable as IFNα upregulates *cxcr4* in HSCs *in vitro* (Tabe et al., 2007). Alternatively, IFNα and chemokines could converge on the JAK/STAT pathway and thus function in a cooperative manner. Clearly, it would be very interesting to determine the downstream targets and resulting cell behaviors of JAK/STAT signaling in stem cell activation, as this knowledge would provide us with additional targets.

Concluding remarks

Although cell migration is a complex process, live imaging and genetic approaches are yielding much information and will continue to do so.

Understanding these processes in genetically tractable model systems will allow deeper understanding of the origin of form and how these mechanisms contribute to human disease. Morphogenic cell migration is a highly dynamic process that can be regulated at the level of acquisition of motility, guidance of directional migration and termination of migration. Experimental interference with any of these processes can lead to aberrant migration and resulting defects in morphogenesis. Live imaging is therefore preferred to examine the precise cellular defects causing such phenotypes. For instance, loss of guidance information can closely resemble loss of motility at a gross phenotypic level. High resolution imaging of cells in fixed samples might reveal the presence of an elaborate cell morphology associated with motility but such processes can be small and short lived and difficult to observe as is the case for *Drosophila* border cells (Prasad and Montell, 2007).

Especially interesting questions that we will have to answer are how adhesion molecules contribute to morphogenic processes. Recent work by Nair and Schilling has demonstrated that chemokines regulate integrin-fibronectin mediated adhesion in migrating endoderm (Nair and Schilling, 2008). These findings raise the possibility that adhesion and guidance might be mechanistically linked in other examples of chemokine mediated migration as well. Additionally, several studies have shown that adhesion molecules not only influence migration via cell-cell adhesion but also via directly regulating the cytoskeleton and protrusion formation.

Also, it is not fully understood how cells migrating in tightly adhering clusters of cells, such as the lateral line primordium, communicate with each other to coordinate their directional migration. Chemokine signaling is required in leading cells but not in trailing cells in the center of the lateral line primordium. As these cells also tumble in the absence of chemokine signaling, they are either mechanically influenced by leading cells or receive, as yet unidentified chemical signals.

Finally, cell migration must be coordinated with other basic cell behaviors such as cell growth, proliferation and shape changes. How these cell behaviors are orchestrated to produce complex three dimensional morphologies remains among the greatest challenges facing modern biology. As misregulation of migration can cause disease, an appreciation of the molecules involved in morphogenic cell migration may also lead to novel therapeutic avenues aimed at the treatment and prevention of cancer invasion and metastasis.

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Figure 4.1. Three steps of cell migration
(A) Prior to migration, cells exhibit a simple morphology and lack protrusions. This is true whether the premigratory cells are part of an epithelium (top) or individual cells (bottom). (B) In the first step of migration, a cell or group of cells (red) begins to elaborate cellular extensions preceding detachment from an epithelium (yellow). Note that a cell of nonepithelial origin, such as zebrafish PGCs, will not need to detach (bottom). In either case, cellular extensions are not initially polarized. Rather, cells extend protrusions in all directions but are still immotile. (C) Cellular extensions are polarized in the direction of migration in response to a gradient of chemoattractant, usually chemokine or growth factor ligands (blue). Cells may also be oriented by repulsive cues (not shown). (C) Cells stop migrating when they lose the ability to elaborate extensions (left) or they reach a region of uniform attractant (left).

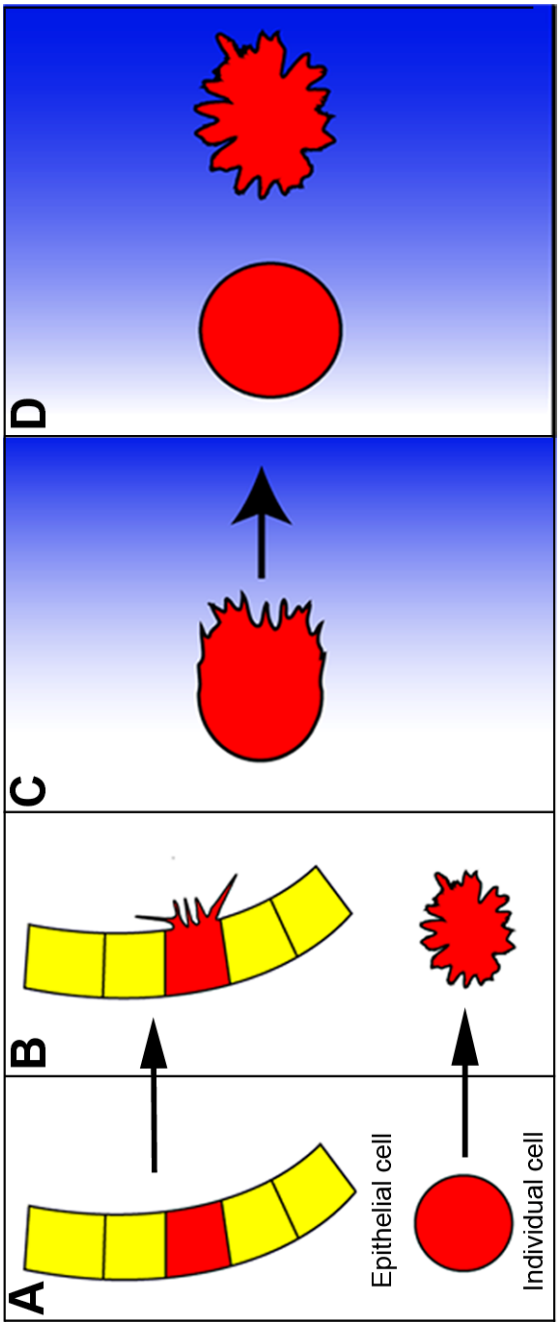
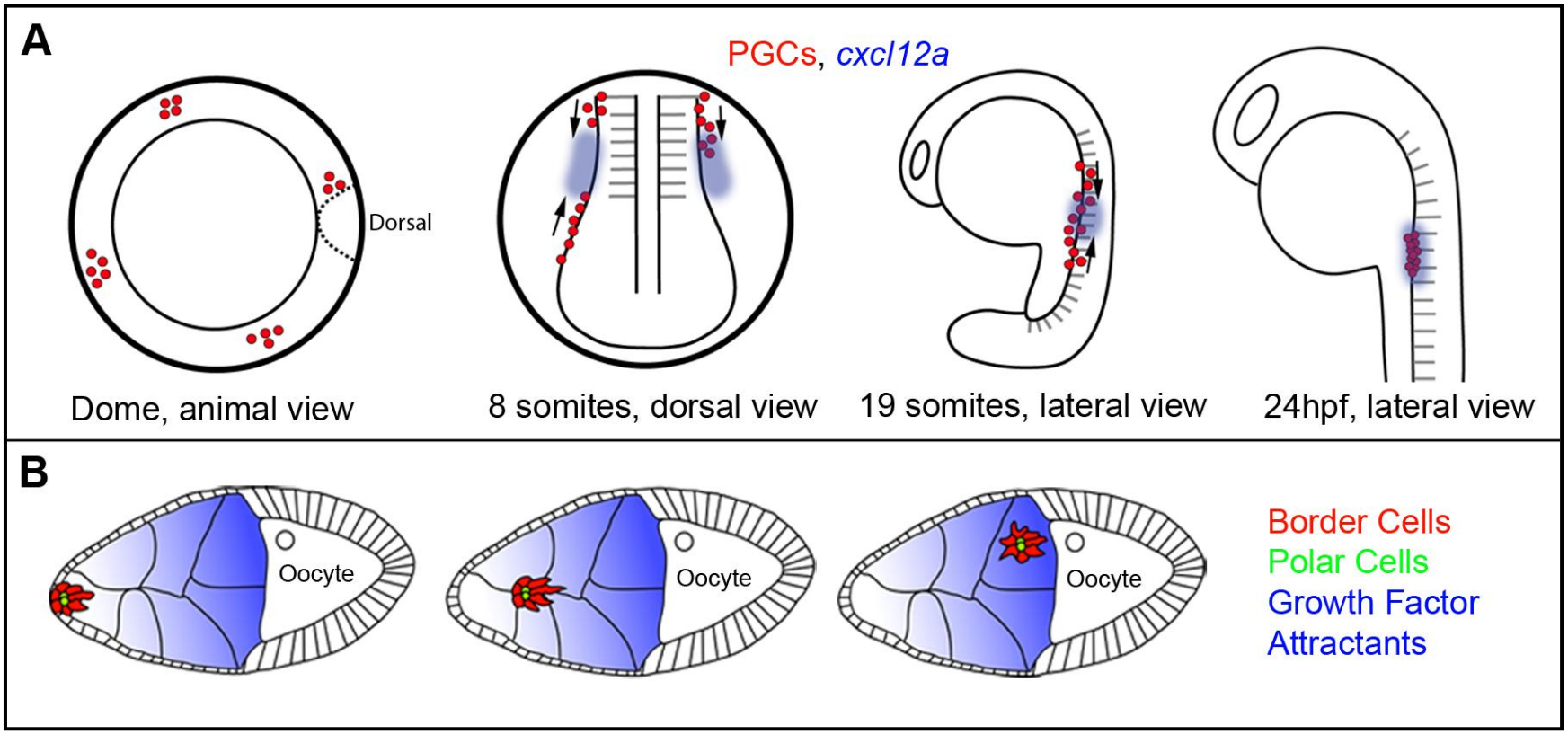

A
B
C
D
Epithelial cell
Individual cell
Phase 1:
No protrusions
Phase 2:
Protrusions with
random orientation
Phase 3:
Protrusions are oriented
toward attractant.
Directional migration begins
Phase 4:
Cells stop migrating due to loss of
protrusions or loss of guidance

Figure 4.2. Examples of *in vivo* cell migration models
(A) Different stages of primordial germ cell (PGC) migration in zebrafish (modified after Raz, 2003). Schematic drawings of embryos from dome stage to 24hpf, which show the positions and movements of PGC clusters. PGCs are represented by small red circles. Arrows indicate the direction of migration. Blue shaded areas mark the expression of the chemoattracant *cxcl12a.* (B) Schematic cross sections through a *Drosophila* egg chamber during border cell migration. Actively migrating border cells (red) form at the anterior pole of the *Drosophila* egg chamber in response to JAK/STAT signaling from the nonmotile polar cells (green). The border cells produce cellular extensions toward the oocyte that depend on gradients of multiple, redundant growth factor ligands (blue) in addition to PCP and Notch signaling (not shown). As border cells approach the oocyte, they occupy a region of uniform growth factor concentration where the cluster loses polarization of protrusions. Subsequently, the border cell cluster stops migrating and forms the micropyle organ to allow sperm entry and fertilization of the egg (not shown).


A
PGCs, cxcl12a
Dorsal
Dome, animal view
8 somites, dorsal view
19 somites, lateral view
24hpf, lateral view
B
Oocyte
Oocyte
Oocyte
Border Cells
Polar Cells
Growth Factor
Attractants

4.3. The zebrafish sensory lateral line system

(A) The fluorescent vital dye DASPEI labels hair cells in the zebrafish lateral line sensory organs. The sensory organs are arranged in lines around the eye and on the trunk. (B) The posterior lateral line placode/primordium migrates as a tight cluster of cells from the ear to the tail tip periodically depositing prosensory organs. (C) Schematic drawing of the lateral line system in the primitive ray-finned fish *Polypterus* (modified with permission from *Brain Behavior and Evolution*). Sensory organs are either situated in bony canals and are connected to the environment via openings or they are located superficially in the skin (grey patches). (D) Schematic representation of primordium migration and sensory organ deposition. The zebrafish lateral line primordium forms just posterior to the otic vesicle (OV) and migrates along a uniform stripe of *cxcl12a* attractant (blue). The migrating lateral line primordium expresses two Cxcl12a binding receptors: *cxcr4a* (red), is expressed in the leading portion of the migrating tissue and is the receptor necessary for guidance toward the tail-tip. *cxcr7b* (green) may not signal in response to Cxcl12a binding and likely is responsible for shaping a gradient of Cxcl12a protein across the length of the primordium. As the primordium migrates, it deposits a series of sensory organ progenitors along the side of the embryo (green rosettes). (E) Cell signaling interaction within the primordium responsible for maintenance of chemokine receptor asymmetry. Solid lines denote genetic interactions and dashed lined denote protein diffusion. Wnt/β-catenin pathway activation in the leading zone (red) leads to Fgf pathway activation in the trailing zone (green). Exclusivity of these domains is maintained by the induction of *dkk1* by Fgf signaling in trailing cells and induction of *sef* by Wnt/β-catenin signaling in leading cells. *cxcr7b* expression in leading cells is inhibited by Wnt/β-catenin signaling, and *cxcr4b* expression is restricted from the trailing zone via the activity of an uncharacterized repressor (R) that is inhibited if Wnt/β-catenin signaling is active throughout the primordium.

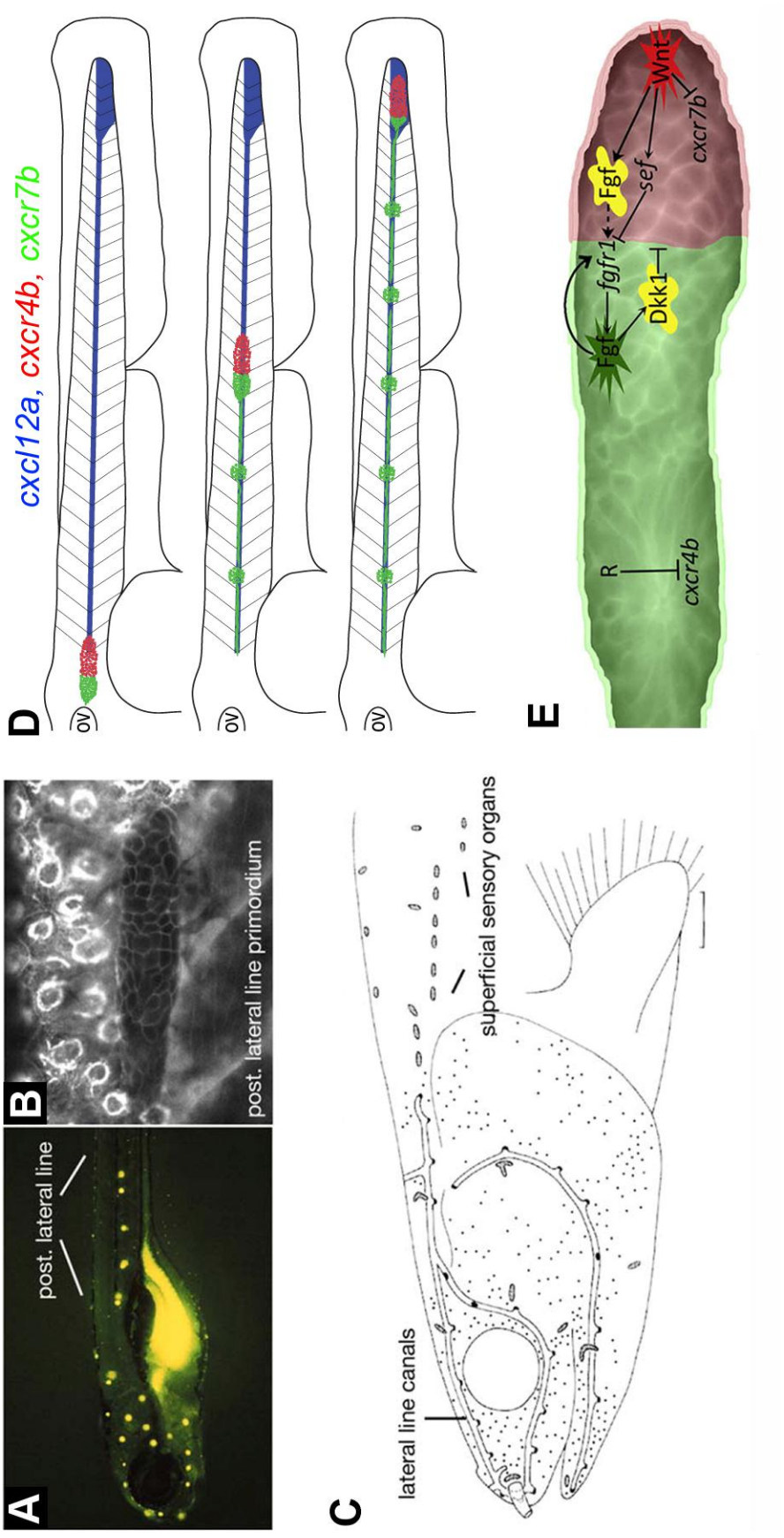

A
post. lateral line
B
post. lateral line primordium
C
lateral line canals
superficial sensory organs
D
cxcl12a, cxcr4b, cxcr7b
ov
ov
ov
E
Wnt
cxcr7b
Fgf
sef
fgfr1
Fgf
Dkk1
R
cxcr4b

Figure 4.4: Migration of angiogenic sprouts, neural crest cells and cells during zebrafish gastrulation

(A) Schematic presentation of retinal angiogenesis in the mouse from perinatal day 1 to 8 (P1 to P8). Sprouting vessels (red) migrate from the central retina toward VEGF-A (blue) expressed in the peripheral retina. Migration occurs concomitantly with endothelial cell proliferation and vessel branching leading to a complex vascular network that covers the entire retina. (A') Schematic of an individual angiogenic sprout migrating up a VEGF-A gradient. Note that only the tip cell (yellow) extends lammelipodia (modified from Gerhardt 2003). (B) Schematic cross section through the trunk of a vertebrate embryo during neural crest migration. Neural crest cells undergo EMT, delaminate from the neural tube and then migrate ventrally along different paths in response to attractive cues (green) and repulsive cues (red) (modified from Tanyhill 2008). (B') High resolution image of GFP labeled migrating neural crest cells *in vivo* connected by thin lamellipodia (arrow heads). Arrow denotes direction of stream migration. (C) Schematic representation of gastrulation movements in zebrafish. (Left) Shield stage. Presumptive mesoderm cells at the margin internalize and actively migrate toward the animal pole (A, red arrows). While the mesoderm migrates directionally the endoderm spreads towards the animal pole via a random walk (blue arrows). These migrations occur across the entire circumference of the blastoderm. (Right) 90% epiboly stage. Internalization and migration of mesoderm continues. Internalized mesoderm and endoderm cells directionally migrate and intercalate dorsally (D), contributing to convergent extension (black arrows; reproduced from Shier and Talbot 2005). (D) Cross section through a *Xenopus* embryo midway through gastrulation. The anterior mesendoderm (AM) expresses PDGFRα and Cxcr4 (red) and migrates toward the blastocoel roof (BCR) in response to the guidance molecules PDGFA and Cxcl12a (blue). Red arrow shows the direction of migration. Abbreviations: BC=Blastocoel, E=Endoderm (modified from Winklbauer et. al. 1996).

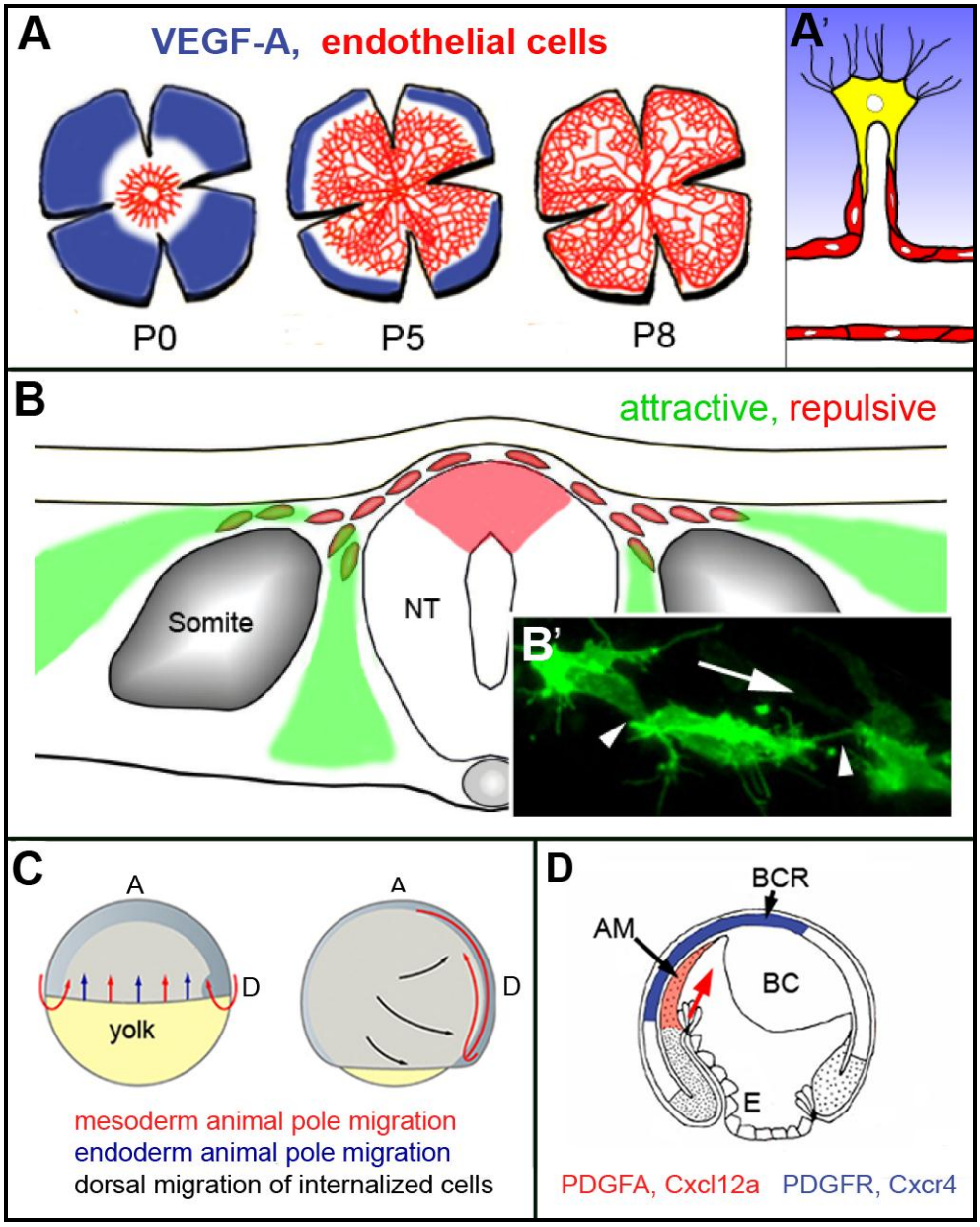

A
VEGF-A, endothelial cells
P0
P5
P8
A'
B
attractive, repulsive
Somite
NT
B'
C
A
D
yolk
A
D
mesoderm animal pole migration
endoderm animal pole migration
dorsal migration of internalized cells
D
BCR
AM
BC
E
PDGFA, Cxcl12a
PDGFR, Cxcr4

Table 4.1. Comparisons of migration regulation between model systems

	I. Protrusion Formation	II. Polarization and Directional Migration	III. Termination of Migration
Vertebrate PGCs	Dead end: vertebrate-specific inhibitor of miRNAs	cxcr4b/7b-cxcl12	uniform cxcl12
Drosophila Border Cells	cytokine-like ligand Unpaired Jak/Stat	Par1 Attractants: Gradient of PDGF-VEGF-like proteins; Receptors: PVI and EGFR Jak/Stat for cohesiveness	uniform growth factors
Vertebrate Lateral Line	unknown	cxcr4b/7b-cxcl12 (likely no gradient)	uniform cxcl12?
Retina angiogenic sprouts	VEGF specifies tip cells	VEGF gradient	fusion with other Vessels, relief of hypoxia leads to loss of VEGF expression
Vertebrate Neural Crest Cells	EMT triggered by BMP, FGF and Wnt that activate snail and foxd3	repellants: robo/slit; neuropilin/semaphorin; ephrin/eph; Wnt/PCP	integration of repellant and attractive signals (eg. ephrinB2 and N-cadherin)

CHAPTER 5

WNT/β-CATENIN DEPENDENT CELL PROLIFERATION UNDERLIES SEGMENTED LATERAL LINE MORPHOGENESIS

The following chapter was written in the form of a peer-reviewed publication and has been submitted for consideration at *Development*. Aman, A., Nguyen, M. Piotrowski, T.

Summary

Morphogenesis is a fascinating but complex and incompletely understood developmental process. The sensory lateral line system consists of relatively few cells and is experimentally accessible making it an excellent model system to interrogate the cellular and molecular mechanisms underlying segmental morphogenesis. The posterior lateral line primordium periodically deposits prosensory organs as it migrates to the tail tip. We demonstrate that periodic neuromast deposition is governed by a fundamentally different developmental mechanism than the classical models of developmental periodicity represented by vertebrate somitogenesis and early Drosophila development. Our analysis demonstrates that cell proliferation and stable gene expression domains in the primordium determine the frequency of prosensory organ deposition. Further, we show that proliferation requires the combined activation of the Wnt/β-catenin and Fgf pathways. We have previously shown that Wnt/β-catenin signaling induces Fgf signaling and that interactions between these two pathways control primordium migration and prosensory organ formation. Here we show that Wnt/β-catenin signaling also controls proliferation and sensory organ deposition, partially via the induction of the transcription factors *hmx2* and *hmx3*. Therefore, by coordinating these different complex cellular behaviors, localized activation of Wnt/β-catenin signaling in the leading leading zone of the primordium orchestrates lateral line morphogenesis.

Introduction

Morphogenesis is a crucial aspect of development. During development, cells arrange themselves into the precise three-dimensional geometries required for function. Although it is widely appreciated that morphogenesis emerges from the coordinated behavior of many cells, understanding morphogenesis in terms of the molecular regulation of basic cell behavior such as proliferation, migration and cell shape changes has proven to be a formidable challenge.

The zebrafish primary posterior lateral line has become a powerful system to interrogate segmental morphogenesis at a cellular and molecular level. The primary posterior lateral line is composed of a series of 5-6 mechanosensory organs called neuromasts distributed along the AP axis of the trunk (Gompel et al., 2001; Metcalfe et al., 1985). These neuromasts develop as immature proneuromasts within a migrating primordium composed of about 100 cells that forms just posterior to the otic vesicle and migrates along the horizontal myoseptum to the tail-tip. The migrating primordium typically houses two or three rosette shaped proneuromasts and periodically deposits them from its trailing end while forming new ones toward the leading end (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). Because the lateral line consists of a metameric series of morphologically similar neuromasts it can be considered a segmented organ system.

While much has been learned about the regulation of primordium migration (Dambly-Chaudiere et al., 2007; Nechiporuk and Raible, 2008; Lecaudey et al., 2008; Aman and Piotrowski, 2008) reviewed in (Aman and

Piotrowski, 2009), little is known about mechanisms regulating the periodic deposition of proneuromasts that underlie the segmented organization of the lateral line. A reduction of Fgf signaling in the primordium leads to deposition of fewer proneuromasts (Nechiporuk and Raible, 2008), however, because Fgf signaling is also necessary for the production of proneuromasts within the primordium it is unclear whether these manipulations are affecting deposition directly or indirectly by slowing the rate of proneuromast formation (Nechiporuk and Raible, 2008; Lecaudey et al., 2008; Aman and Piotrowski, 2008).

We have previously shown that a cell signaling feedback network within the primordium stably subdivides the tissue into two distinct domains (Aman and Piotrowski, 2008) . Wnt/β-catenin signaling is responsible for inducing and localizing Fgf signaling to the trailing portion of the primordium, which in turn restricts Wnt/β-catenin signaling to the leading region. The localized activation of Wnt/β-catenin signaling maintains the polarized transcription of the two chemokine receptors *cxcr4b* and *cxcr7b* which is crucial for directed, collective migration of primordium cells (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Here we demonstrate that the compartmentalization of Wnt/β-catenin and Fgf signaling is not only important for primordium migration but is also crucial for generating periodicity of sensory organ deposition. Because Wnt/β-catenin signaling regulates primordium migration and proneuromast formation, manipulations that directly affect Wnt/β-catenin signaling in the primordium are not suitable to study the mechanism of periodic deposition. We have functionally dissected the downstream cellular outputs of Wnt/β-catenin signaling and studied

the effect of cell addition to the primordium using manipulations that do not affect primordium migration and proneuromast formation.

Studies by the Ghysen lab have postulated that cell proliferation plays a crucial role in the formation of the lateral line, as the migrating primordium contains approximately 100 cells and the completed primary lateral line comprises approximately 200 cells (Gompel et al., 2001; Laguerre et al., 2005; 2009). However, the exact nature of how proliferation affects morphogenesis of the lateral line or the nature of the underlying signaling pathways has not been explored. Our results show that unlike classical examples of segmentation, such as vertebrate somitogenesis and early Drosophila development, periodicity of proneuromast placement is controlled by the rate of cell proliferation. By manipulating signaling within the primordium and assaying proliferation we demonstrate that Wnt/β-catenin and Fgf signaling combine to induce cell proliferation in the leading and central regions of the primordium causing the tissue to continuously lengthen, displacing proneuromasts toward the trailing edge. To determine the effect of cell proliferation on the segmental morphogenesis of the lateral line we treated embryos with pharmacological cell cycle inhibitors and analyzed mutant embryos characterized by an increase in apoptosis. These methods directly affect the rate of cell addition to the primordium without affecting migration and proneuromast formation. These approaches revealed that a reduction in the rate of cell addition leads to a decrease in the frequency of proneuromast deposition resulting in fewer, but nevertheless normally sized proneuromasts.

This segmentation mechanism is interesting in that it relies on two continuous processes, stable compartmentalization and steady proliferation, with no apparent input from molecular oscillators. Importantly, our analysis demonstrates that Wnt/β-catenin signaling in the leading region of the primordium coordinates three crucial cell behaviors in the migrating lateral line primordium, thus orchestrating segmental morphogenesis of the sensory lateral line. Wnt/β-catenin controls primordium migration via the regulation of chemokine receptors, it induces proneuromast formation via the upregulation of the Fgf pathway and it controls periodic proneuromast deposition via the regulation of proliferation.

Results

Proneuromast deposition is regulated by a primordium autonomous mechanism independent of underlying segmented somite soundaries

Based on candidate gene expression in somite boundaries, it has been proposed that signals from somite boundaries trigger proneuromast deposition (Haines et al., 2004). To test this possibility we analyzed proneuromast distribution in *trilobite (tri)* mutant embryos which, because of a defect in convergent extension movements, have shorter somites than wildtype (wt) embryos (Sepich et al., 2000). We reasoned that if proneuromast deposition was triggered by somite boundaries then proneuromasts would be more closely spaced in mutants with shortened somites, such as *tri*. On the other hand, if

proneuromast deposition was regulated by a primordium autonomous mechanism the distance between proneuromasts would be similar between wt and *tri* mutants, irrespective of the number of somite boundaries traversed.

For this analysis, the distance between the first and second proneuromast (named L1 and L2) was measured in wt and *tri* homozygous mutants. The actual distances in μm as well as the number of somites between L1 and L2 were counted (Figure 5.1). This analysis revealed that primordia migrate over significantly more somites between proneuromast depositions in *tri* mutants (Figure 5.1C; 6.7±1.3 somites in wt (n=46); 11.3±2.3 somites in *tri;* n=32; p=2.9E-13). However, the actual distance between proneuromasts is not significantly different (Figure 5.1D; 162±20μm in wt, n=22; 199±50μm in *tri,* n=58; Student's t-test: p=0.32). This experiment demonstrates that proneuromast deposition is not triggered by traversing a set number of somite boundaries and that it is likely regulated by a primordium-autonomous mechanism.

A decrease in cell proliferation in the primordium leads to deposition of fewer proneuromasts

As the primordium does not contain enough cells at the onset of migration to deposit five to six primary proneuromasts, cell proliferation is necessary for the formation of a complete posterior lateral line. Here we tested whether cell proliferation affects the frequency of proneuromast deposition or whether the periodicity of proneuromast deposition is cell cycle-independent. If proliferation is important for periodicity of proneuromast deposition we predict that embryos with a slowed cell cycle would deposit fewer, normally sized proneuromasts. On the

other hand, if cell proliferation is only necessary to maintain progenitor cells within the primordium, such a manipulation would yield a truncated lateral line with normally spaced proneuromasts in the anterior trunk and an absence of proneuromasts further posterior.

To reduce proliferation in wt primordia we soaked the embryos in various doses of the DNA replication inhibitors aphidicolin and hydroxyurea (APH; Figure 5.2A-D; Krokan et al., 1981). At the doses classically used to inhibit the cell cycle completely, we observed a simultaneous increase in cell death (not shown). As we aimed to analyze the effect of proliferation in the absence of cell death, we soaked the embryos in low doses of APH that did not cause any cell death. In these experiments proliferation is not abolished but rather the cell cycle length appears increased as cells accumulate in S phase, yielding primordia with a greater proportion of cells in S phase revealed by BrdU incorporation experiments (Figure 5.2C).

By lowering the rate of cell addition using APH we observed that primordia deposited significantly fewer proneuromasts (Figure 5.2B,D,F; 3.9±0.54 proneuromasts in APH treated embryos, n=10; 4.9±0.49 proneuromsts in DMSO treated embryos, n=10; Student's t-test p=4.0E-6). These few proneuromasts are distributed along the trunk of the animal and terminal proneuromasts are apparent, demonstrating that the primordium was able to complete its normal migration. Conversely, we predict that accelerating proliferation would lead to an increase in neuromast number. Unfortunately, there are currently no tools available to test this hypothesis.

To confirm that the deposition phenotype observed in APH treated embryos is due to a suppression of the cell cycle and not another effect of the reagents, embryos were treated with the CDK2 inhibitor olomoucine (Figure 5.2E,F; Alessi et al., 1998; Veselý et al., 1994). Similar to APH, doses of olomoucine that effectively abolish progression through the cell cycle also show a large amount of cell death preventing migration. However, the minimum dose at which no cell death is apparent leads to a dramatic reduction in proneuromast numbers confirming that proliferation is important for periodicity of proneuromast deposition (Figure 5.2F; 2.0±0.0 proneuromasts in olomoucine treated embryos, n=4; Student's t-test p=2.5E-16 vs. wt). Surprisingly, although treatment with olomoucine leads to a strong reduction in the number of deposited proneuromasts and therefore reduced the number of cells added to the lateral line over the course of treatment (Figure 5.2F), this has no apparent effect on BrdU index (BrdU index for DMSO treated embryos is 0.22±0.04, n=10; 500μM olomoucine treated embryos is 0.26±0.07, n=10; Student's T-test p=0.13). This is likely due to the capacity of olomoucine to suppress several phases of the cell cycle (Alessi et al., 1998; Schutte et al., 1997). In mild doses like the one used here cell cycle progression is suppressed, but not eliminated, yielding a tissue with a normal proportion of cells in S phase at any given time.

Cell death in the primordium leads to deposition of fewer proneuromasts

In a second set of experiments that lowered the rate of cell addition we analyzed proneuromast distribution in *bap28* homozygous mutants, which exhibit

elevated apoptosis in proneural tissue including the lateral line primordium (Figure 5.2G,I; Azuma et al., 2006). Wt primordia do not exhibit any signs of apoptosis (Figure 5.2G). Many *bap28* mutant embryos display a truncated lateral line due to premature stalling of the primordium similar to the phenotype caused by a mutation in *eya1*/*dog-eared* that is also characterized by cell death in the primordium (data not shown; Kozlowski et al., 2005). We hypothesize that primordia must maintain a certain size in order to sustain polarized chemokine receptor expression and directional collective migration. In order to be able to study proneuromast deposition we restricted our analysis to mutant individuals with primordia that had reached the tail tip and completed migration by 54hpf. Our analysis revealed that a wt primordium deposits 5.4±0.50 proneuromasts along its journey to the tail-tip (Figure 5.2H,O; n=22). In contrast, *bap28* mutant embryos show a marked decrease in proneuromast number relative to wt embryos (Figure 5.2J,O; 2.3±1.1 proneuromasts; p=2.5E-14 vs. wt; n=24 *bap28* homozygotes).

In addition to inducing cell death genetically, cell death in the nervous system can also be induced as a toxic, off target effect of some morpholinos (Robu et al., 2007). We discovered that injection of a *tcf7* MO targeting the translation start site leads to randomly distributed apoptosis in the primordium causing a significant decrease in the number of deposited proneuromasts (Figure 5.2K,L,O; *tcf7* morphants 3.6±0.70 proneuromasts; p=2.2E-16 vs. wt; n=40). Importantly, the reduction in proneuromast number in *tcf7* MO injected embryos is nonspecific and not due to loss of *tcf7* function, as *tcf7* maternal/zygotic null

mutants (*tcf7mz*) show no sign of apoptosis and exhibit no deposition defects (Figure 5.2M,N,O; = 5.3±0.80 proneuromasts in *tcf7* mutants; p=0.63 vs. wt; n=40; Nagayoshi et al., 2008).

It is imperative to note that *tcf7* morphant primordia migrate at the same rate as wt primordia (Figure 5.3A-C; position of wt primordia at 32hpf = 16.0±1.1somites, n=29; *tcf7* morphant position = 16.3±1.4 somites, n=27; Student's t-test p=0.44). Proneuromasts deposited from such primordia contain the same number of cells as wt proneuromasts (Figure 5.3A-B,D; wt proneuromasts harbor 26.1±3.4 cells; *tcf7* morphant proneuromasts harbor 24.2±4.0 cells; Student's t-test p=0.10). Additionally, the strong induction of cell death observed in *bap28* mutants does not lead to any compensatory increase in proliferation (Figure 5.3E-G; wt sib BrdU index = 0.26±0.10, n=6; *bap28* BrdU index = 0.26±0.10, n=9; Student's t-test p=0.998).

To confirm that cell death is causing proneuromast deposition defects we inhibited cell death in *bap28* mutants and *tcf7* morphants by injection of a *p53* MO. This morpholino suppresses the p53-dependent apoptosis pathway and rescues some morpholino off target effects (Haines et al., 2004). Indeed, injection of *p53* MO significantly rescues neuromast deposition in *bap28* mutant and *tcf7* morpholino injected embryos (Figure 5.2O; *bap28-/-* +p53MO have 4.3±0.90 proneuromasts; n=24; p=8.1E-14 vs. uninjected mutants; *tcf7* ATG MO+ *p53* MO have 4.4±0.60 proneuromasts; n=28; p=3.0E-7 vs. *tcf7* ATG MO alone). The analysis of these different manipulations demonstrates that cell death leads to the deposition of fewer but normally sized proneuromasts. Since

migration speed is unaffected compared to *tcf7* ATG MO injected embryos, these primordia have the same amount of time to generate and deposit proneuromasts. We conclude that the rate of net cell addition to the primordium influences the frequency of proneuromast deposition.

Primordium length fluctuates during the deposition cycle

The discovery that the net rate of cell addition determines how often proneuromasts are deposited suggests that a critical primordium length might trigger proneuromast deposition. Because in our hands *tcf7* ATG MO toxicity leads to the most reliable mild induction of apoptosis and subsequent reduction in proneuromast deposition without impairing migration, we chose to focus our analysis on these embryos. We measured the lengths of *Tg(claudinb:GFP)* and *Tg(claudinb:GFP) tcf7* ATG MO injected primordia during four defined phases of the deposition cycle using timelapse analyses. Each timelapse movie encompassed two deposition cycles and lasted approximately 10h (data not shown**;** n=4 and n=7 for wt and *tcf7* ATG MO injected embryos, respectively). The analyses of *Tg(claudinb:GFP)* embryos revealed a periodic lengthening of primordia preceding proneuromast deposition followed by a shortening of the tissue after proneuromast deposition (Figure 5.4A-D,I). Interestingly, primordia with increased apoptosis in *tcf7* ATG MO injected embryos only deposit proneuromasts after reaching similar lengths as wt primordia (Figure 5.4E-H, I). As net cell addition is slowed in morphant primordia, they migrate over a longer distance than wt primordia before they reach the critical length at which proneuromast deposition is initiated. We therefore conclude that primordium

length influences when a proneuromast is deposited. This observation supports the hypothesis that cell proliferation influences the rate of periodic proneuromast deposition.

The Wnt/Fgf feedback system maintains a deposition domain at a stable distance from the leading edge irrespective of the deposition cycle and primordium length

To understand how periodic primordium lengthening contributes to periodic proneuromast deposition we analyzed gene expression in primordia in various phases of the deposition cycle. Previous work in our laboratory elucidated a feedback circuit whereby Wnt/β-catenin and Fgf signaling maintain two distinct gene expression domains within the primordium. In the leading zone, Fgf signaling is reduced to a very low level via the inhibition by Wnt/β-catenin signaling and in the trailing zone, Wnt/β- catenin signaling is inhibited by Fgf signaling (Aman and Piotrowski, 2008). An important consequence of this feedback system is that Wnt/β-catenin signaling in the leading zone of the primordium represses the chemokine receptor *cxcr7b* and thus restricts its expression to the trailing portion of the tissue. Interestingly, *cxcr7b* is expressed in cells that face imminent deposition (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). It is currently not understood if *cxcr7b* is actively involved in proneuromast deposition or if it merely serves as a marker for the region of slowing and depositing cells. Unfortunately, the analysis of a possible role for *cxcr7b* in proneuromast deposition is complicated by the fact that *cxcr7b* appears to be invovled in primordium migration precluding analysis of proneuromast

deposition (Dambly- Chaudiere et al., 2007; Valentin et al., 2007; Aman and Piotrowski, 2008; Nechiporuk and Raible, 2008).

As we aimed to understand how primordium length correlates with proneuromast deposition, we used *cxcr7b* expression as a marker for the deposition zone. We analyzed the extent of the *cxcr7b* expression domain with respect to the overall length of the primordium as well as the *cxcr7b*-free zone during various phases of the deposition cycle (Figure 5.5A,B). We observed that the length of the *cxcr7b* expression domain correlates with and fluctuates by about the same amount as the length of the entire primordium (Figure 5.5C; coefficient of determination *R2*=0.80). This result suggests that in a longer primordium more cells are *cxcr7b* positive and begin to be deposited. In contrast, the length of the *cxcr7b*-free zone remains relatively static, while the length of the primordium fluctuates by about 30% as proneuromasts are formed and deposited (Figure 5.5D). This analysis demonstrates that the Wnt/Fgf feedback system maintains a deposition zone at a defined distance from the tip of the primordium. As new cells are added to the primordium through proliferation, existing proneuromasts are progressively displaced into this deposition zone where they slow down and are ultimately deposited.

Importantly, *cxcr7b* expression does not fluctuate within cells in the primordium in a time-dependent manner as would be expected for an oscillating gene. Rather, expression of this gene is dependent on where cells are positioned within the primordium. Individual cells do not begin expressing *cxcr7b* until they are displaced into the trailing domain beyond the inhibitory influence of Wnt/β-

catenin signaling. However, proneuromasts are only deposited once the entire proneuromast rosette has entered the *cxcr7b* domain, thus ensuring periodicity of proneuromast deposition.

Primordium growth is regulated by the combined activity of Wnt/β-catenin and Fgf signaling

As wt primordia do not exhibit appreciable levels of apoptosis and the lateral line system is primordium derived (Figure 5.2G; Grant et al., 2005), cell addition and proneuromast deposition is entirely dependent on cell proliferation. We therefore analyzed cell proliferation patterns and signaling pathways that could be involved in regulating proliferation in the primordium thereby regulating the rate of proneuromast deposition.

A 32hpf wt primordium consists of a leading unpatterned region and two to three proneuromasts depending on the deposition cycle. It was previously described that proliferation occurs in the leading two-thirds of the primordium, whereas the trailing one-third is relatively quiescent. To corroborate and further extend these studies, we analyzed patterns of proliferation in the leading zone and the third proneuromast before deposition in wt embryos. The leading zone possesses a relatively high mitotic index whereas the trailing-most rosette is significantly less proliferative (Figure 5.6A,C). Therefore, based on their proliferative behavior, wt primordia can be divided into two distinct regions, as previously reported (Hava et al., 2009; Laguerre et al., 2005). The primordium migrates approximately four or five somites between proneuromast depositions (Laguerre et al., 2005). Therefore, we can infer the phase of the deposition cycle

in fixed samples by measuring the distance between the trailing edge of the primordium and the most recently deposited proneuromast (Laguerre et al., 2009). A primordium that is located four or five somites away from the last deposited proneuromast is about to deposit another proneuromast, and is therefore called a predeposition primordium (Figure 5.6D). A primordium located only one to two somites away from a proneuromast is called a postdeposition primordium. As the postdeposition primordium has just deposited the third proneuromast, it is shorter and only contains two rosettes (Figure 5.6D). We determined the BrdU indexes for long pre deposition and short postdeposition primordia. Our analyses demonstrate that longer primordia that possess a quiescent third rosette have a relatively lower BrdU index compared to shorter primordia that only have two rosettes (Fig 6D,F; BrdU index for postdeposition primordia = 0.32±0.05, n=30; BrdU index for predeposition primordia = 0.26±0.05, n=29; Student's t-test p= 6.3E-6). It was previously proposed that the BrdU index fluctuates in the trailing one-third of the primordium (Laguerre et al., 2005)**.** However, this study did not take into account that the primordium fluctuates in length during the deposition cycle periodically losing the third, relatively quiescent rosette.

Interestingly, the relative restriction of BrdU incorporation to the leading zone of the primordium correlates with the portion of the tissue with active Wnt/β-catenin signaling (Figure 5.5A,B; 4.6B,E). This correlation suggests that Wnt/β-catenin signaling might be mitogenic in the primordium as in other developing

organs and certain cancers (Haramis et al., 2006; Kuhnert et al., 2004; You et al., 2006; Bonner et al., 2008; Megason and McMahon, 2002).

To test whether Wnt/β-catenin signaling is required for proliferation in the primordium, we downregulated Wnt/β-catenin signaling by heat shocking *Tg(hs:Dkk1)* embryos (Stoick-Cooper et al., 2007). This manipulation leads to a significant reduction in proliferation in the primordium (Figure 5.6G-I; heatshocked non transgenic siblings, BrdU index = 0.25±0.06, n=21; heatshocked transgenic embryos, BrdU index = 0.10±0.03, n=25, p=2.7E-11). This result demonstrates that Wnt/β-catenin signaling is necessary for proliferation to occur. Conversely, constitutive activation of the Wnt/β-catenin pathway throughout the length of the primordium in *apcmcr* mutant embryos leads to a high level of proliferation throughout the tissue (Figure 5.6J-L; Hurlstone et al., 2003). The level of proliferation throughout *apcmcr* primordia is similar to that observed in the leading zone of wt primordia where Wnt/β-catenin signaling is active (Figure 5.5A-C; 6A-C,JL; BrdU index = 0.34±0.06 in *apcmcr*, n=28; BrdU index = 0.33±0.11 in wt leading zone, n=14, p=0.49). Additionally, primordia that have just deposited a proneuromast and therefore have a proportionally shorter region free of Wnt/β-catenin signaling have a similar BrdU index as *apc* mutant primordia (Student's t-test p=0.23). Thus, Wnt/β- catenin signaling drives proliferation at its maximum in the wt leading domain and in *apcmcr* embryos this domain is expanded in the trailing direction.

Even though these manipulations of the Wnt/β-catenin pathway using *Tg(hs:Dkk1)* or *apc* mutant embryos show that Wnt/β-catenin is required for

proliferation, there is the caveat that the expression of Fgf ligands depends on Wnt/β- catenin signaling in the primordium (Aman and Piotrowski 2008). Thus, manipulations that affect the distribution of Wnt/β-catenin signaling will also affect the distribution of Fgf signaling (Figure 5.6H,K). To unequivocally determine whether Wnt/β-catenin signaling is sufficient to induce proliferation in the primordium we inhibited Fgf signaling with the Fgf receptor inhibitor SU5402, which causes simultaneous expansion of Wnt/β-catenin signaling (Figure 5.6N; Aman and Piotrowski, 2008). If Wnt/β-catenin signaling was the sole regulator of proliferation, this manipulation would be expected to lead to an expansion of the proliferative leading zone similar to *apc* mutants.

Surprisingly, Fgf inhibition strongly reduces proliferation in the primordium and distinct domains are no longer detectable (Figure 5.6M-O; BrdU index for DMSO treated embryos = 0.26±0.06; n=28; BrdU index for 5μM SU5402 treated embryos = 0.04±0.02; n=18; p=6.7E-19). To confirm that the effect is due specifically to loss of Fgf signaling and not an off-target effect of SU5402 treatment, embryos harboring an inducible dominant negative Fgfr1 construct were analyzed. Induction of dn-Fgfr1 also leads to significant reduction of proliferation (Figure 5.7; BrdU index for heatshocked nontransgenic siblings=0.28±0.07; n=18; BrdU index for heatshocked transgenic embryos =0.21±0.06; n=16; p= 0.005). This effect is weaker than SU5402 treatment likely due to the transient expression of heatshock-induced transgenes compared to continuous drug treatment. The analysis of Fgf signaling depleted primordia revealed that Wnt/β-catenin signaling is not sufficient to induce proliferation.

To test if Fgf signaling is sufficient to induce proliferation in the absence of Wnt/β- catenin signaling we determined the mitotic index in heatshocked *Tg(hs:ca-fgfr1)* embryos in which the Fgf pathway is constitutively active (Marques et al., 2008). Heatshock induction of ca-Fgfr1 is effective as revealed by the upregulation of tyrosine phosphorylation causing the simultaneous loss of Wnt/β-catenin signaling (Figure 5.8). In primordia expressing constitutively active *fgfr1* the BrdU index is significantly lower than in wt primordia demonstrating that in the absence of Wnt/β-catenin, Fgf signaling is not sufficient to induce high levels of proliferation (Figure 5.6P-R; BrdU index for heatshocked, non-transgenic sibs = 0.23±0.05, n=15; BrdU index for heatshocked *Tg(hs:ca-fgfr1)* embryos = 0.09±0.04, n=15; Student's t-test p=1.2E-8).

In conclusion, normal rates of proliferation require the combined activity of both the Fgf and Wnt/β-catenin signaling pathways. Indeed, in wt primordia proliferation is highest in the region of overlap between these pathways (Figure 5.6A,B,E,F). We have previously described that the Wnt/β-catenin target *lef1* is expressed in the very anterior unpatterned leading region of the primordium and that Fgf signaling is not active in that domain (Aman and Piotrowski, 2008). As we lose proliferation in the *lef1* zone in the absence of Fgf signaling (Figure 5.6 M-O), Fgf signaling has to have an effect on that region, even though *pea3*, a transcriptional target of Fgf signaling is absent (Aman and Piotrowski, 2008). Indeed, *fgfr1* is expressed at very low levels in the leading region and it is possible that low levels of Fgf signaling influences proliferation through a different transcription factor than *pea3* (Nechiporuk and Raible, 2008). Alternatively, an

Fgf dependent factor expressed in the posterior of the primordium could diffuse anteriorly where it regulates proliferation together with Wnt/β-catenin dependent target genes. Similarly to the Fgf pathway, Wnt/β-catenin pathway activation is not restricted to the most anterior *lef1* expression domain. Other Wnt/β-catenin target gene expression, such as *fgf10* and *axin2* are expressed further into the trailing region overlapping with Fgf signaling (Aman and Piotrowski 2008).

Interestingly, the uniformity of proliferation in the leading zone of the primordium suggests that Wnt/β-catenin and Fgf signaling are not dose dependent mitogens in this context. The unpatterned leading zone experiences the highest level of Wnt/β-catenin signaling and the lowest level of Fgf signaling (Figure 5.6B; Aman and Piotrowski 2008). Nevertheless, the BrdU index in this region is not significantly lower than in the leading two rosettes which experience high levels of both signaling activities as revealed by expression of target genes (data not shown; (Aman and Piotrowski, 2008). In *apc* mutant embryos the region of overlap between Wnt/β-catenin and Fgf pathway activation expands to fill the entire tissue, resulting in primordia that exhibit a uniform high rate of proliferation, whereas in *Tg(hs:Dkk1)* embryos that lack both signaling activities, proliferation is strongly reduced (Figure 5.6G-I).

<u>The primordium proliferation regulators *hmx2 a*nd *hmx3 a*re Wnt/β-Catenin targets</u>

hmx2 and *hmx3* are members of the Hmx transcription factor family and are strongly expressed in the lateral line primordium (Figure 5.9A; Feng and Xu, 2010). *hmx* genes act redundantly and are involved in vertebrate inner ear

development, especially via the regulation of cell proliferation (Hadrys et al., 1998; Wang et al., 2000; 2004). In zebrafish, morpholino knockdown of *hmx2* and *hmx3* abolishes proliferation without inducing apoptosis leading to small lateral line primordia that fail to initiate migration (Feng and Xu, 2010). This failure of migration prevented us from analyzing the deposition of proneuromasts. However, we asked whether *hmx2* and *hmx3* are regulated by the Fgf or Wnt/β-catenin pathway and if loss of these genes is responsible for the lack of proliferation in Fgf or Wnt/β-catenin signaling depleted embryos. In 32hpf wt embryos, the expression domains of *hmx2* and *hmx3* in the primordium are identical. Here we only show expression of *hmx2.* Both genes are expressed throughout the leading zone of the primordium. Expression in the most trailing region is much weaker or absent (Figure 5.9A). In *apc* mutant embryos, in which the Fgf and the Wnt/β-catenin pathways are upregulated, *hmx2 and hmx3* are uniformly expressed in all cells of the primordium suggesting that these genes might indeed be regulated by these pathways (Figure 5.9B). To test if *hmx2 and hmx3* expression depends on Wnt/β-catenin signaling in the absence of Fgf signaling we heatshocked *Tg(hsDkk1)* embryos at 26hpf and fixed them at 32hpf. *hmx2 and hmx3* expression is drastically reduced demonstrating that Wnt/β-catenin signaling is an important regulator of their expression (Figure 5.9C). On the other hand, treatment with the Fgfr1 inhibitor SU5402 revealed that the Fgf pathway does not regulate *hmx2 and hmx3* expression, as previously described (Figure 5.9D; Feng and Xu, 2010). In SU5402 treated embryos *hmx2* and *hmx3* expression is ubiquitously strong, as Wnt/β-catenin is upregulated in these

primordia. Even though *hmx2* and *hmx3* are expressed, SU5402 treated primordia do not proliferate demonstrating that *hmx2* and *hmx3* are not sufficient to induce proliferation in the absence of other Fgf dependent factors (Figure 5.6M-O, 4.7D). We initially considered a rescue experiment by injecting RNA encoding *hmx2* or *hmx3* into Wnt/β- catenin signaling depleted embryos to test if *hmx2* and *hmx3* are the main downstream effectors of Wnt/β-catenin controlling proliferation in the primordium. However, downregulation of Wnt/β-catenin signaling by heatshock induction of Dkk1 also abolishes Fgf signaling (Figure 5.6H; Aman and Piotrowski, 2008). Injection of *hmx2* or *hmx3* mRNA into these embryos would therefore copy the SU5402 experiment in which the presence of *hmx2* and *hmx3* is not sufficient to drive proliferation in the absence of Fgf signaling (Figure 5.9D).

Combined these experiments show that Wnt/β-catenin dependent *hmx2* and *hmx3* are required but not sufficient to induce proliferation in the primordium.

Discussion

The proliferation-dependent primordium lengthening model of proneuromast deposition

The regulation of periodicity and segmental morphogenesis in development is a fascinating problem. The two best understood mechanisms for generating periodicity are early Drosophila development and vertebrate somitogenesis. Saturated genetic screens have revealed a detailed mechanism by which the Drosophila embryo is segmented. Briefly, periodicity in the Drosophila embryo is initiated by gradients of maternally provided morphogens.

The distribution of morphogens leads to a complex cascade of transcriptional regulators that establish and refine domains of spatially periodic gene expression that ultimately underlie the segmented adult body plan. Periodicity in this context is generated by combinatorial transcriptional activation and repression. This process is extremely rapid and occurs simultaneously along the axis of the embryo (reviewed in Pick, 1998).

In contrast, vertebrate somitogenesis is driven by a substantially different mechanism. During somite formation, waves of synchronized gene expression pass through the presomitic mesoderm such that individual cells will display oscillating gene expression with the period of oscillation exactly matching the period of somite formation. In this context, periodicity is generated by the instability and autorepressive activity of Hairy/E(spl) proteins. Because these proteins repress their own transcription and are intrinsically unstable, their expression can oscillate with a reliable period (reviewed in Kageyama et al., 2009). This periodicity is synchronized across neighboring presomitic mesoderm cells by the action of Delta-Notch signaling, allowing for the propagation of periodic waves of gene expression (reviewed in Lewis et al., 2009). Importantly, proliferation plays no role during these segmentation mechanisms. Drosophila segmentation occurs while the embryo is in a nonproliferative syncytial state, and periodicity of somite formation occurs normally in embryos that lack cell proliferation. Indeed, proliferation leads to disturbances in coordinated oscillation of individual cells during somite formation and therefore introduces a certain amount of noise (Zhang et al., 2008).

The lateral line is also a segmented tissue, as metameric proneuromasts are repeated along the axis of the embryo. However, the findings of the present work suggest that the periodic organization of proneuromasts is generated by a fundamentally different mechanism that depends crucially on continuous cell proliferation and migration (Figure 5.10). During migration, cells in the leading zone of the primordium proliferate leading to a progressive lengthening of the tissue (Figure 5.10; green domain). Because proliferation is uniform in the leading zone of the primordium, proneuromasts within the primordium occupy more and more trailing positions during primordium migration. In support of this model, lineage-tracing studies revealed that cells initially occupying the extreme leading edge of the primordium proliferate to give rise to daughter cells that occupy increasingly trailing positions within the primordium and ultimately reside in deposited proneuromasts (Nechiporuk and Raible, 2008). We suggest that this displacement of cells is driven by cell proliferation in the leading portion of the primordium. Ultimately, the trailing-most proneuromast rosette is entirely displaced into the deposition zone labeled by expression of *cxcr7b* (Figure 5.10; blue domain). Once a proneuromast occupies the *cxcr7b* positive deposition domain it begins to slow down and is deposited leading to a shortened primordium and initiating a new cycle. Our data have demonstrated that both the placement of the *cxcr7b* positive deposition zone and the rate of cell proliferation depend on Wnt/β-catenin signaling. Therefore, Wnt/β-catenin signaling represents a crucial coordinator of lateral line morphogenesis, linking proliferation with deposition.

This model represents a novel mechanism for generating periodicity in the embryo. Unlike *Drosophila* segmentation and vertebrate somitogenesis, segmentation of the lateral line relies crucially on dynamic cellular behaviors during migration. The period of proneuromast deposition is determined by the rate of cell proliferation, as shown by the analyses of embryos in which cell proliferation rates are experimentally reduced or that show an increase in apoptosis. In these embryos the rate of proneuromast deposition is reduced. This mechanism is capable of generating periodicity based on stable, compartmentalized gene expression domains and cell proliferation, and does not require the activity of molecular oscillators.

Wnt/β-catenin signaling controls morphogenesis by integrating migration, proliferation, proneuromast formation and deposition

Morphogenesis depends on the precise orchestration of several complex cellular behaviors. Our analysis of cell migration, proliferation and periodic proneuromast deposition reveal that these developmental processes are co-regulated and integrated via the localized activation of Wnt/β-catenin signaling in the leading zone of the primordium. We have previously shown that spatially restricted Wnt/β-catenin signaling is crucial for collective migration of the primordium (Aman and Piotrowski, 2008). In addition, Wnt/β-catenin signaling induces Fgf-dependent proneuromast formation (Aman and Piotrowski, 2008; Nechiporuk and Raible, 2008; Lecaudey et al., 2008). Here we demonstrate that the same Wnt/β-catenin signal controls periodic proneuromast deposition by establishing the boundary of the deposition domain and, together with Fgf

signaling, controlling the rate of cell proliferation that displaces proneuromasts into the deposition domain. Therefore, Wnt/β-catenin signaling is a crucial coordinator of morphogenesis in the lateral line that couples migration with sensory organ development and positioning.

hmx2 and *hmx3* are required for proliferation in the lateral line primordium and we tested if the loss of proliferation in Wnt/β-catenin and Fgf depleted primordia is caused by a loss of *hmx2* and *hmx3* (Feng and Xu, 2010). It was previously shown that *hmx2* and *hmx3* expression is independent of Fgf signaling and indeed in SU5402 treated embryos *hmx2* and *hmx3* are still expressed. On the other hand, *hmx2* and *hmx3* depend on Wnt/β-catenin signaling, as their expression is drastically reduced in heatshocked *Tg(hs:Dkk1)* embryos. As *hmx2* and *hmx3* are not sufficient to induce proliferation in the absence of Fgf signaling, we conclude that *hmx2* and *hmx3* act as transcriptional co-activators with other Fgf-dependent factors to induce proliferation. It is interesting to note that migration and periodic deposition must be coordinated to ensure the correct number and spacing of proneuromasts. While the rate of cell proliferation determines the period of proneuromast deposition, the speed of migration determines how many periods can occur. We have shown that decreasing the rate of cell addition by inducing low level apoptosis in the primordium is capable of increasing this period without affecting migration speed, leading to the deposition of fewer proneuromasts. We hypothesize that manipulations that reduce the rate of primordium migration without affecting proliferation rates would increase the number of deposited proneuromasts by increasing the total time

primordia have to complete deposition cycles. Unfortunately, there are currently no tools available that allow us to manipulate migration speed without inducing cell death or affecting cell proliferation. However, we were able to induce cell death without affecting migration speed (Figure 5.3C) demonstrating that these two cellular behaviors are independently regulated. Our data support the view that cell proliferation rate determines the period of the deposition cycle and the migration speed determines the total amount of time available to complete deposition cycles. It will be interesting to examine how cell proliferation rates and/or primordium migration speed differ between zebrafish and other aquatic vertebrates that deposit many closely spaced neuromasts, such as Xenopus (Winklbauer, 1989)**.** We hypothesize that in Xenopus either the proliferation rate or the primordium migration speed have increased. Elucidating the molecular mechanisms underlying morphogenesis in the zebrafish thus allows us to investigate how changes in these mechanisms can lead to the evolution of different organ shapes.

The complex interactions and feedback loops between different signaling pathways in the developing lateral line are reminiscent of the well-described pathway interactions underlying limb and tooth development (ten Berge et al., 2008)**.** As such complex interactions surely underlie most biological processes, studies that focus solely on the effects of individual signaling pathways may have to be reevaluated. For example, we show here that proliferation in the primordium depends not only on Wnt/β- catenin but also on Fgf signaling. Wnt/β-catenin signaling has previously been shown to be important for regulating

proliferation many different tissues including tumors. It now remains to be determined if Wnt/β-catenin signaling is sufficient to drive proliferation in these tissues, or whether input from other signaling pathways is also important. Answering these questions is particularly important if the aim of these studies is to design therapeutic treatments, for example to inhibit tumor growth. The careful examination of signaling networks that lie downstream of pleiotropic morphogens, such as Wnts and Fgfs, may reveal less toxic and more efficacious therapeutic approaches.

The cellular basis of proneuromast deposition

Although the present study has not focused on a role for adhesion molecules in proneuromast deposition, it is important to note that differential adhesion must be crucial for the generation of periodicity in the lateral line. Rosette-shaped proneuromasts are always deposited as whole entities and they therefore present cohesive units of tissue separation (Hava et al., 2009). In the absence of these, as yet unidentified adhesion molecules, cells would be lost continuously. We speculate that interneuromast cells that give rise to postembryonic neuromasts and that are deposited as strings of cells in between proneuromasts likely lack these particular adhesion molecules (Grant et al., 2005; Lopez-Schier and Hudspeth, 2005). In addition, although the entire migrating primordium is quite cohesive along its journey, the rosettes are able to detach from its trailing edge. These observations suggest that proneuromasts may express adhesion molecules that are different in composition or concentration from those that mediate cohesion within the bulk of the migrating

primordium. The nature of adhesion molecules expressed in the primordium and their regulation remain to be elucidated. Because the trailing rosette represents a gene expression domain characterized by the lack of Wnt/β-catenin signaling and expression of *cxcr7b,* it is tempting to hypothesize that expression or function of adhesion molecules may be regulated by the Wnt/β-catenin /Fgf feedback system discussed above. In this scenario, the lack of Wnt/β-catenin signaling in the trailing rosette may endow cells in this domain with distinct adhesive properties. Alternatively, deposition could be driven by *cxcr7b* expression, even though, at least in zebrafish primordial germ cells, Cxcr7b does not transduce signals like Cxcr4b**.** Instead, Cxcr7b binds and internalizes Cxcl12a (formerly called Sdf1a), sequestering it from interacting with the guidance receptor Cxcr4b (Boldajipour et al., 2008; Naumann et al., 2010). These findings suggest the hypothesis that *cxcr7b* expression in the trailing region masks these cells from sensing Cxcl12a and that cells in this region stop migrating and deposit from the primordium due to a loss of guidance information (Aman and Piotrowski, 2009)**.** Unfortunately, since *cxcr7b* loss of function by morpholino injection disrupts primordium migration, this hypothesis is difficult to test with available techniques (Valentin et al., 2007; Dambly-Chaudiere et al., 2007).

Regardless of the cellular mechanism underlying deposition, this work shows that periodic deposition is sensitive to the rate of cell addition to the primordium and that cell addition is driven by the coordinated activity of Wnt/β-catenin and Fgf signaling. Future work must now determine what special

properties are present in the *cxcr7b*-positive deposition zone that causes proneuromasts to deposit from the primordium.

Materials and methods

Fish strains

The following fish strains were employed: *Tg(Cldnb:lynGFP),* referred to as *Tg(Claudinb:GFP)* in the text. (Haas and Gilmour, 2006). *apcmcr* (Hurlstone et al., 2003), *Tg(hs:Dkk1)* (Stoick-Cooper et al., 2007), *Tg(hsp70l:dnfgfr1-EGFP)pd1, referred to as Tg(hs:dn-fgfr1)* in the text (Lee et al., 2005), *Tg(hsp70:ca-fgfr1) referred to as Tg(hs:ca-fgfr1)* in the text, *trilobite (trim209/m209)* (Sepich et al., 2000). *tcf7mz* mutant embryos were derived from a cross of homozygous mutant parents (Nagayoshi et al., 2008) and *bap28-/-* (Azuma et al., 2006).

In situ hybridization

Stainings were performed as described (Kopinke et al., 2006). In situ probes used: *eya1*(Sahly et al., 1999)*; klf4* (Kawahara and Dawid, 2000)*; cxcr7b* (Dambly-Chaudiere et al., 2007); *lef1* (Dorsky et al., 2003); *pea3* (Münchberg et al., 1999). Fragments of *hmx2* and *hmx3* were amplified from mixed stage cDNA using primers (hmx2F:CTGGAAAGGACAGTCCCAAA; hmx2R:TCTCTCGGAGCTGCTCAAAT; hmx3F:GCCTATTTTGGCACCCACTA; hmx3R:CCATTTGTTTCTGCGGTTCT) and cloned into pCRII-TOPO. Fragments were sequenced to confirm fidelity and orientation. Antisense in situ probes were generated by amplifying the cloned fragments using M13 primers. The resulting

product was cleaned using a QiaexII Gell Extraction Kit (Qiagen) and transcribed with SP6 RNA polymerase (Roche).

Morpholino injections

Morpholinos used: 2nL of a 1.2mM MO *tcf7-xatg:* 5'-AGCTGCGGCATGATCCAAACTTTCT-3' (Gene Tools LLC; gift from R. Dorsky). 2nL of a 0.022mM solution *p53*: 5'-GCGCCATTGCTTTGCAAGAATTG-3' solution was injected (Langheinrich et al., 2002). Morpholinos were diluted in 0.1 M KCl and 5% phenol red.

BrdU assays

BrdU incorporation was performed as described in (Laguerre et al., 2005). Briefly, dechorinated embryos were soaked in 15% DMSO in E3 and 15mM BrdU (Sigma) for 30 minutes on ice, washed three times with E3 and placed at 28.5ºC for 1 hour. Embryos were fixed in 4% PFA overnight at 4ºC and stored in methanol at -20ºC. For fluorescent immunostaining embryos were rehydrated and treated with 0.02mg/ml proteinaseK in PBS + 0.1% Tween20 (PBST) for 5 minutes. Embryos were then washed three times in PBST and fixed for 30 minutes at room temperature (RT) in 4% PFA. Fixed embryos were washed three times with PBST and then twice with deionized water. Embryos were placed in a solution of 2N HCl in water for 1 hour at RT, washed three times with PBST and blocked for 1 hour at RT in a solution of 10% newborn goat serum and 1% DMSO in PBST. Mouse anti-BrdU was used at a dilution of 1:400 overnight at 4ºC (Roche). Alexa fluor 568 goat anti-mouse secondary antibody (Invitrogen) was used to visualize BrdU at a dilution of 1:400. To visualize primordium nuclei

embryos were placed in a solution of 0.1ng/ml DAPI (Invitrogen) for 2 hours at RT or 0.1ng/ml DAPI was added to the secondary antibody. Stained embryos were visualized using a Zeiss LSM5 Live or a Zeiss LSM710 confocal microscope. BrdU indices were calculated as the ratio of BrdU+ nuclei to all DAPI stained nuclei.

TUNEL assay

Apotag Red In Situ Apoptosis Detection Kit was used as per manufacturer's instructions (Millipore). The staining was followed by DAPI incubation and imaging as described above.

SU5402 Treatment

SU5402 (Calbiochem; gift from M. Brand; Mohammadi et al., 1997) was diluted to 5μM in E3 medium containing 1% DMSO. Dechorionated embryos were incubated from 22- 32hpf and fixed in 4% PFA. Loss of *pea3* expression was used to confirm loss of Fgf signaling in the primordium (Aman and Piotrowski, 2008).

Pharmacological inhibition of proliferation

To suppress progression through S phase dechorinated embryos were placed in a mixture of 75μM aphidocolin/7.5mM hydroxyurea (APH) in E3 containing 1% DMSO (Sigma). To suppress progression from G1 through G2 embryos were placed in 500uM olomoucine (Enzo Life Sciences). Higher doses of these reagents lead to apoptosis (data not shown).

Heatshock induction of transgenes

Heterozygous fish were crossed to wt animals. Offspring were incubated at 42ºC for 20 minutes followed by 20 minutes at RT and for an additional 20 minutes at 42ºC. 50% of the embryos did not carry the transgene and served as a control. Effective inhibition of Wnt/β-catenin signaling was confirmed by loss of *lef1* expression from the primordia of heatshocked *Tg(hs:Dkk1)*. Effective inhibition of FgfR1 was confirmed by loss of *pea3* in heatshocked *Tg(hs:dn-fgfr1)* embryos. Effective activation of Fgf signaling in heatshocked *Tg(hs:ca-fgfr1)* embryos was confirmed by the upregulation of *pea3* (not shown) and increased abundance of phosphorylated tyrosine (detected with mouse anti-phospho-tyrosine at 1:400 (Millipore, Cat# 05-321X) and standard antibody staining procedures.

Timelapse microscopy

Embryos were anesthetized and mounted in 0.8% low melting point agarose in E3 medium. Z-stack recordings were made on inverted Zeiss LSM710 or Nikon A1R confocal microscopes with a stage-top incubator using 10x or 40x objectives and manipulated using ImageJ.

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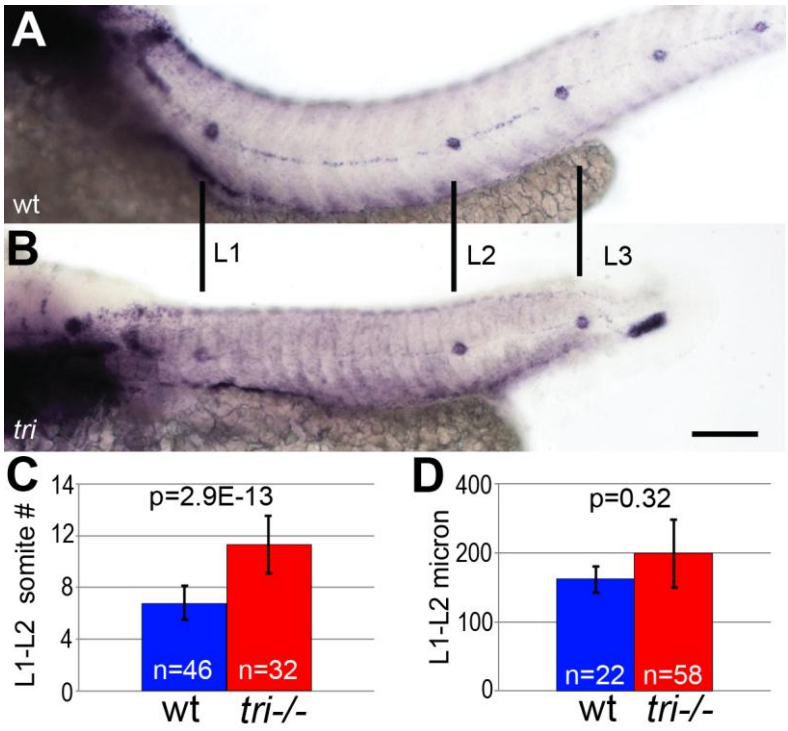


Figure 5.1. Proneuromast deposition is not triggered by traversing somite boundaries

(A,B) *eya1* in situ hybridization labels deposited proneuromasts. (A) A wt embryo with a typical distribution of proneuromasts. (B) A *tri* homozygous mutant displays similarly spaced proneuromasts, even though somites are narrower. (C) Quantification of somites between the first two proneuromasts in wt and *tri* mutants. Mutant primordia cross significantly more somite boundaries between proneuromast depositions in *tri* mutants. (D) The actual distance between these proneuromasts in wt and *tri* mutants is not significantly different. Scale bar equals 50μm. Error bars represent standard deviation from the mean.

Figure 5.2. Increasing apoptosis leads to deposition of fewer proneuromasts
(A,C,E) BrdU positive cells are labeled red and *Tg(claudinb:GFP)* labels membranes green. (B,D,F,H,J,L,N) Primary lateral line morphology revealed by *klf4* in situ hybridization. Proneuromasts are numbered and the terminal proneuromasts are labeled with a 'T'. Presence of terminal proneuromasts demonstrates completed primordium migration. (A) Primordia treated with DMSO from 26-32hpf proliferate normally. (B) DMSO treated primordia migrate and deposit a normal complement of proneuromasts along the trunk. (C) Treatment with 75μM aphidicolin + 7.5mM hydroxyurea (APH) from 26-32hpf causes cells to stay longer in S phase leading to an accumulation of BrdU positive cells with no increase in membrane blebbing and nuclear fragmentation associated with cell death. (D) APH treatment from 26-54hpf leads to a significant reduction in the number of deposited proneuromasts without preventing primordium migration. (E) Treating embryos from 26-32h with the CDK2 inhibitor olomoucine has no apparent effect on BrdU labeling in the primordium, and does not cause cell death. (F) Treatment with 500μM olomoucine from 26-54hpf leads to a strong reduction in proneuromast deposition. (G,I,K,M) Whole mount TUNEL assay at 32hpf. Nuclei are labeled with DAPI and red spots represent TUNEL signal. Dying cells are rapidly extruded and form a cloud surrounding and trailing behind the migrating primordium. (G) No appreciable apoptosis is detectable in a wt primordium. (H) A typical 48hpf wt primary lateral line. (I) *bap28* homozygous mutants show a dramatic increase in apoptosis. (J) *bap28* mutants possess fewer primary proneuromasts but still produce terminal proneuromasts. (K) Injection of toxic *tcf7* ATG MO leads to apoptosis in the primordium. (L) *tcf7* ATG MO also causes deposition of fewer proneuromasts, though to a lesser extent than *bap28* mutants. (M) Maternal/zygotic *tcf7* null mutants show no apoptosis in the primordium. (N) *tcf7* mutants have a normal number of primary proneuromasts. (O) Quantification of proneuromast number. Manipulations that decrease the rate of cell proliferation or increase apoptosis lead to deposition of fewer proneuromasts. This effect of apoptosis can be rescued by co-injection of *p53* MO. Error bars equal standard deviation. Scale bars equal 50μm.

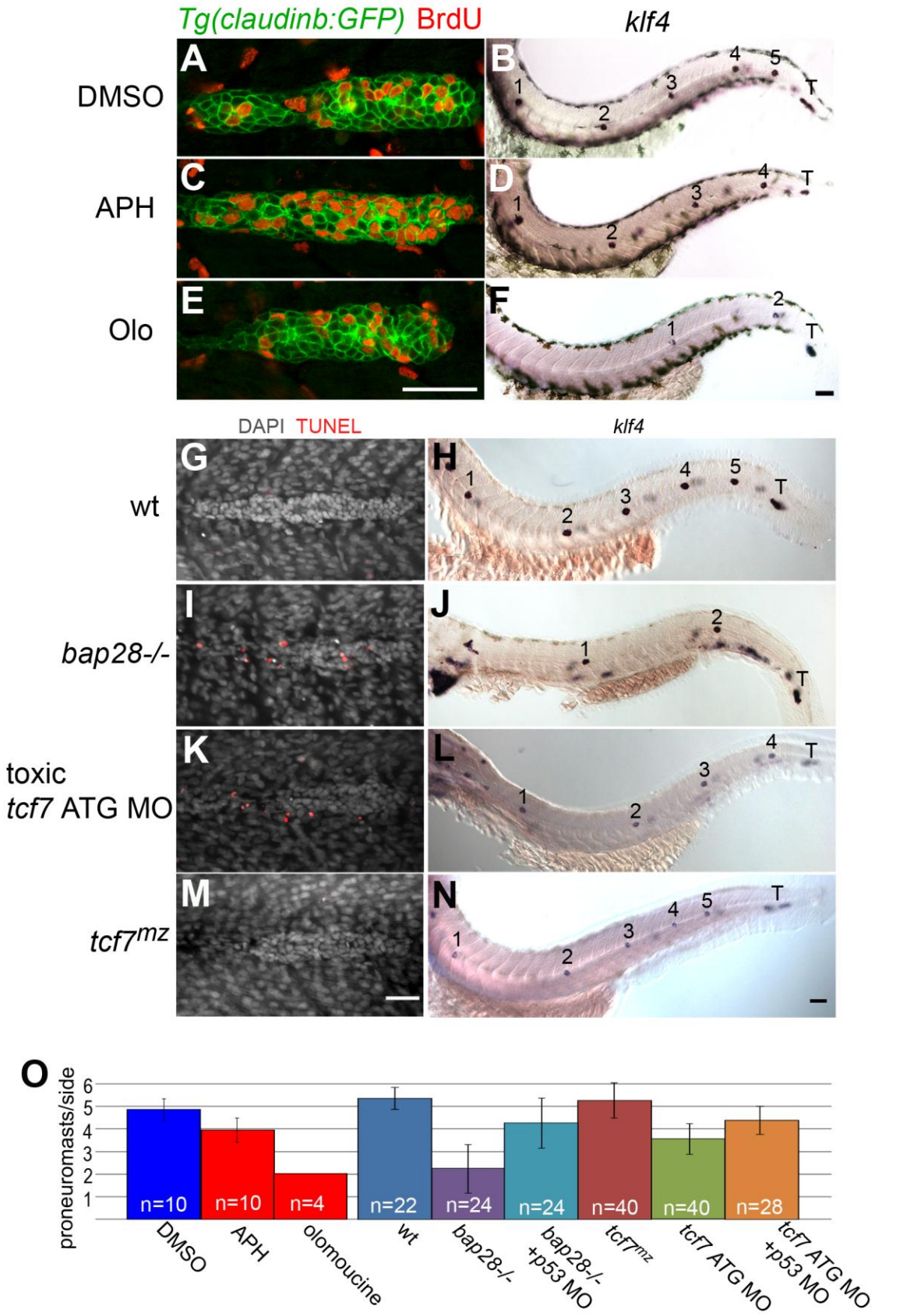

Tg(claudinb:GFP) BrdU
klf4
DMSO
APH
Olo
DAPI TUNEL
klf4
wt
bap28-/-
toxic
tcf7 ATG MO
tcf7mz
O
proneuromasts/side
n=10
n=10
n=4
n=22
n=24
n=24
n=40
n=40
n=28
DMSO
APH
olomoucine
wt
bap28-/-
bap28-/- +p53 MO
tcf7mz
tcf7 ATG MO
tcf7 ATG MO +p53 MO

Figure 5.3. Apoptosis does not interfere with primordium migration, proneuromast deposition or proliferation

(A,B) primordia (within white ovals) and proneuromasts (within white rectangles) stained with DAPI (grey) at 32hpf. (A) Wt primordium. (B) t*cf7* morphant primordium. (C) Somite positions of the leading edge of wt primordia (blue) and *tcf7* morphant primordia (red) at 32hpf are not significantly different, demonstrating that migration speed is normal in injected embryos. (D). Wt and apoptotic *tcf7* morphant primordia deposit proneuromasts that are not significantly different in cell number.(E) BrdU labeling (red) in wt and (F) *bap28* mutant primordia, counterstained with DAPI (grey). (G) The BrdU indexes of wt and *bap28* mutant primordia are not significantly different.

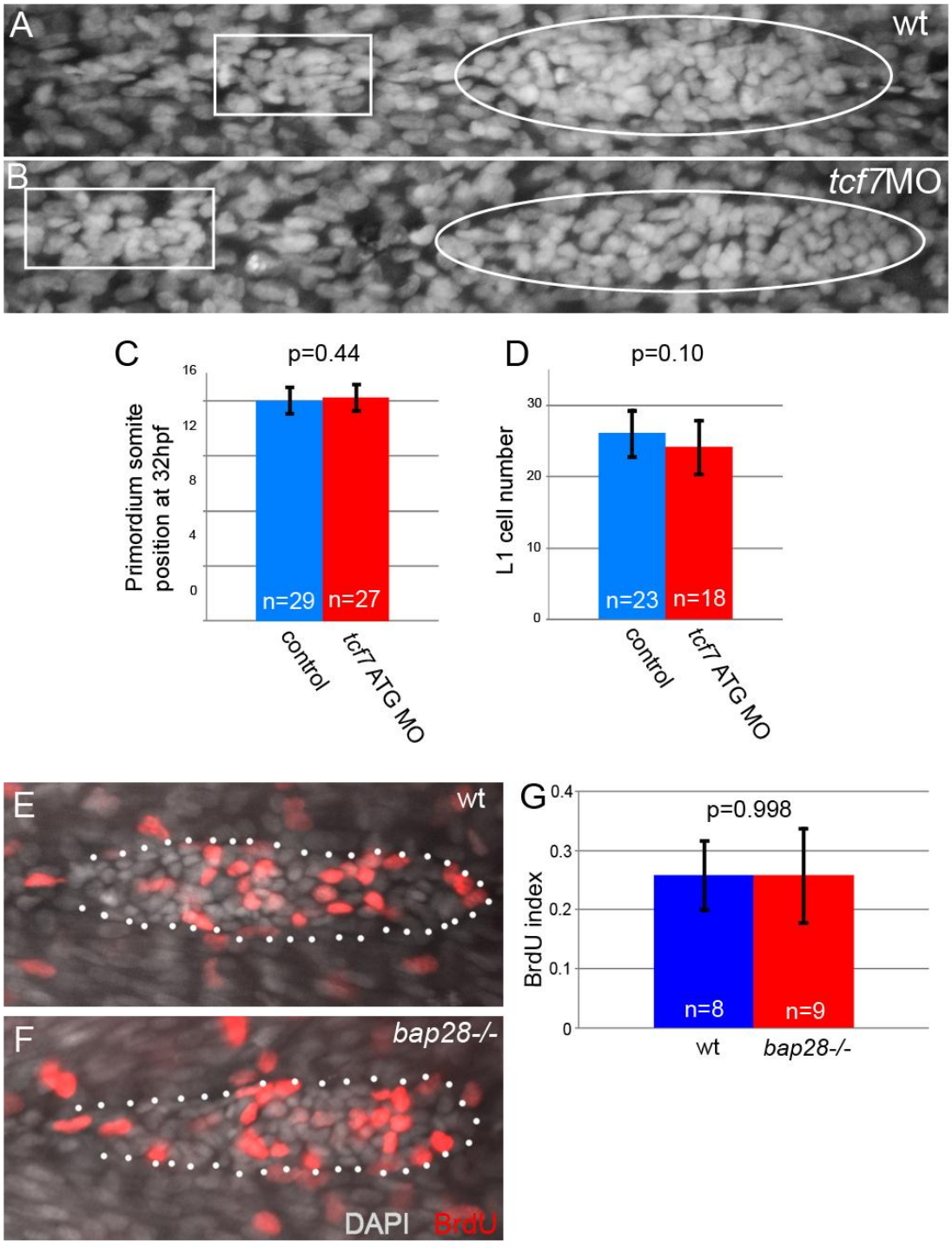
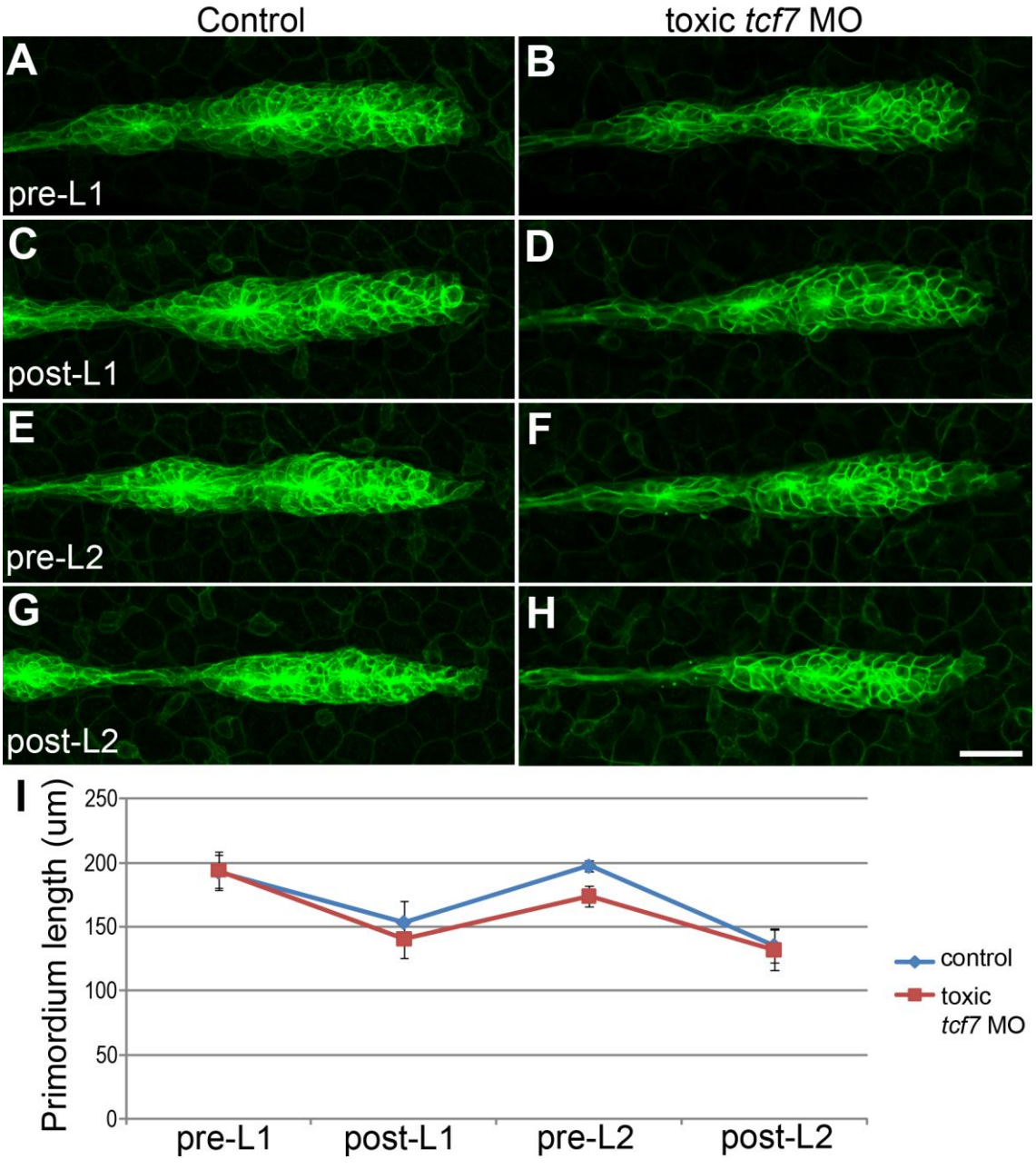

A
wt
B
tcf7MO
C
p=0.44
Primordium somite position at 32hpf
16
12
8
4
0
n=29
n=27
control
tcf7 ATG MO
D
p=0.10
L1 cell number
30
20
10
0
n=23
n=18
control
tcf7 ATG MO
E
wt
F
bap28-/-
DAPI
BrdU
G
p=0.998
BrdU index
0.4
0.3
0.2
0.1
0
n=8
n=9
wt
bap28-/-

Figure 5.4. Primordium lengthening precedes proneuromast deposition
Still images of timelapse movies of proneuromast deposition in control and *tcf7* MO injected*Tg*(*claudinb:GFP)* transgenic embryos (A-H). (A-D) Wt primordia in different phases of the deposition cycle. (A) Wt primordium immediately prior to deposition of the first primary proneuromast (L1). (B) The same primordium immediately following deposition of L1. (C) Wt primordium preceding deposition of the second proneuromast (L2). (D) Wt primordium following deposition of L2. (E,F) *tcf7* morphant primordia before deposition and following deposition of L1. (G,H) The same primordium immediately before and following deposition of L2. (I) Quantification of primordium lengths in wt embryos (blue, n=4) and *tcf7* morphants (red, n=7). Wt and *tcf7* morphant primordia achieve similar lengths preceding deposition of L1 (A,E,I). Wt and *tcf7* morphant primordia shrink to similar sizes following deposition of L1 (B,F,I). *tcf7* morphant primordia are slightly shorter than wt primordia preceding L2 deposition (C,G,I). However, following deposition of L2, wt and *tcf7* morphant primordia again possess the same length (D,H,I).


Control
toxic *tcf7* MO
A
B
pre-L1
C
D
post-L1
E
F
pre-L2
G
H
post-L2
I
Primordium length (um)
250
200
150
100
50
0
pre-L1
post-L1
pre-L2
post-L2
control
toxic *tcf7* MO

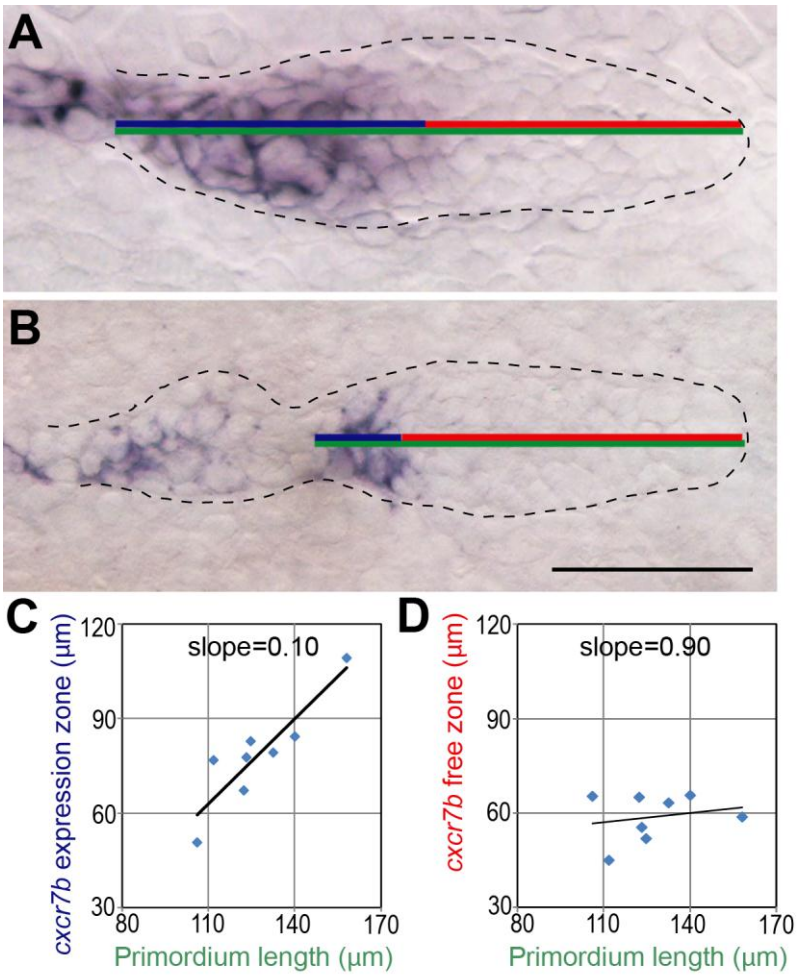
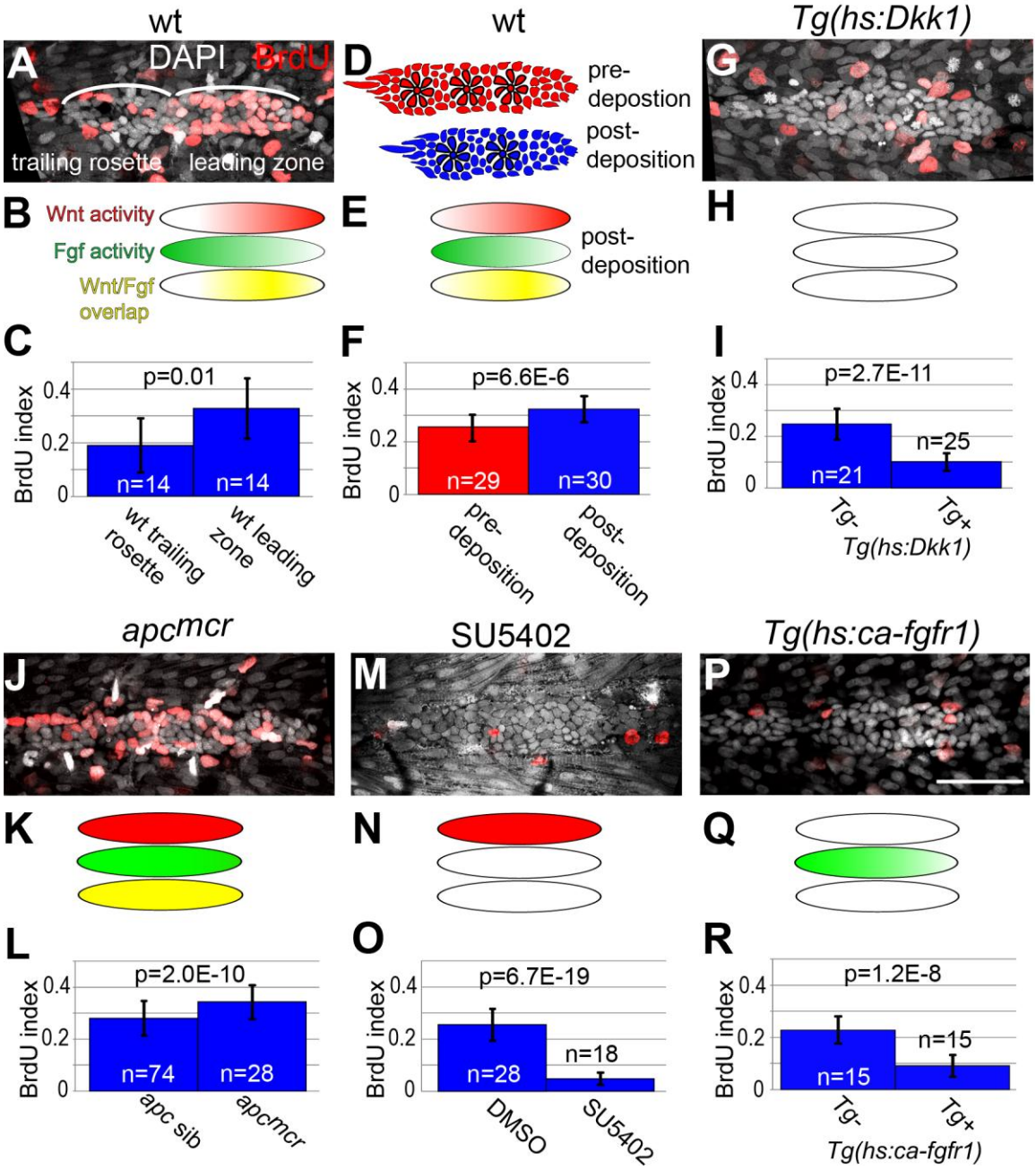


Figure 5.5. *cxcr7b* expression begins at a constant distance from the leading edge

(A,B) *cxcr7b* in situ hybridization labels the trailing portion of the primordium. (A) Example of a long primordium that is about to deposit a proneuromast. Such long primordia possess a relatively wide *cxcr7b* expression domain. (B) A primordium that has just deposited a proneuromast. Most of the *cxcr7b* expressing cells were deposited yielding a short primordium with a relatively narrow *cxcr7b* expression domain. Total length (green), length of the *cxcr7b* expression domain (blue), and the length of the *cxcr7b*-free domain (red) were measured in primordia during various phases of the deposition cycle (n=8). (C) The length of the *cxcr7b* expression domain fluctuates with primordium length. Longer primordia about to deposit have longer *cxcr7b* expression domains (slope =0.9; R_2=0.80). (D) The length of the *cxcr7b* free zone remains relatively constant as primordium size fluctuates (slope =0.1). Scale bar equals 50µm.

Figure 5.6. Wnt/β-catenin and Fgf signaling synergistically control proliferation in the primordium

(A,G,J,M,P) BrdU labeling in the primordium (red). Embryos were given a one hour pulse of BrdU at 32hpf and immediately fixed. Nuclei are stained with DAPI (grey). (B,E,H,K,N,Q) Schematic representations of the distribution of Wnt/β-catenin signaling (red), Fgf signaling (green), and the region of overlap between these pathways (yellow). (C,F,I,L,O,R) Quantification of BrdU indexes for wt, *Tg(hs:Dkk1), apc* and SU5402 treated primordia. (A) Wt primordium with the leading zone and trailing rosette marked. Note the relatively low level of BrdU in the trailing rosette. (B) Schematic representation of Wnt/β-catenin and Fgf signaling activity in predeposition wt primordia. (C) Quantification of proliferation in the leading zone and trailing rosette of predeposition wt primordia. BrdU index is higher in the leading zone of wt primordia relative to the trailing rosette. (D) Schematic representation of a pre-deposition primordium harboring three rosettes (red) and a postdeposition primordium harboring two rosettes (blue). (E) Postdepositon primordia shorter than predepoition primordia and have a smaller region free of wnt/β-catenin signaling. (F) Postdeposition primordia have a significantly higher BrdU index compared to predeposition primordia due to the loss of the relatively quiescent trailing rosette. (G) BrdU in a *Tg(hs:Dkk1)* primordium. Embryos were heatshocked six hours prior to BrdU incorporation. (H) Induction of Dkk1leads to loss of Wnt/β-catenin and Fgf signaling in primordium. (I) Induction of Dkk1 significantly reduced BrdU. (J) *apc* mutant embryo. BrdU labeling is homogeneous throughout the mutant primordium. (K) *apc* mutation leads to the activation of both the Wnt/β-catenin and Fgf pathways throughout the primordium. (L) *apc* mutant primordia have a significantly higher BrdU index compared to wt sibs. The BrdU index in *apc* mutant primordia is similar to the index in the leading zone of wt primordia (compare (A) and (J)). (M) An embryo treated with 5 μM SU5402 from 22-32hpf. (N) In SU5402 treated embryos Wnt/β-catenin signaling activity expands throughout the primordium. (O) The Fgf inhibitor SU5402 significantly reduces the BrdU index. (P) Induction of constitutively active Fgf receptor by heatshocking *Tg(hs:ca-fgfr1*) embryos reduces BrdU incorporation in the primordium. (Q) *ca-fgfr1* expression leads to increased stimulation of Fgf signaling and a subsequent loss of Wnt/β-catenin signaling. (R) *cafgfr1*expression leads to a significant reduction in BrdU incorporation.


wt
A
DAPI
BrdU
trailing rosette
leading zone
B
Wnt activity
Fgf activity
Wnt/Fgf overlap
C
p=0.01
BrdU index
0.4
0.2
0
n=14
n=14
wt trailing rosette
wt leading zone
wt
D
pre-depostion
post-deposition
E
post-deposition
F
p=6.6E-6
BrdU index
0.4
0.2
0
n=29
n=30
pre-deposition
post-deposition
Tg(hs:Dkk1)
G
H
I
p=2.7E-11
BrdU index
0.4
0.2
0
n=21
n=25
Tg-
Tg+
Tg(hs:Dkk1)
apcmcr
J
K
L
p=2.0E-10
BrdU index
0.4
0.2
0
n=74
n=28
apc sib
apcmcr
SU5402
M
N
O
p=6.7E-19
BrdU index
0.4
0.2
0
n=28
n=18
DMSO
SU5402
Tg(hs:ca-fgfr1)
P
Q
R
p=1.2E-8
BrdU index
0.4
0.2
0
n=15
n=15
Tg-
Tg+
Tg(hs:ca-fgfr1)

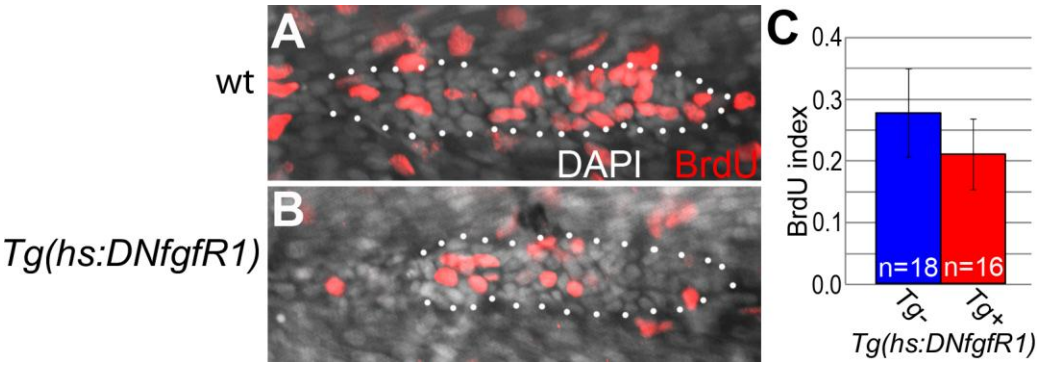


Figure 5.7. Reducing Fgf signaling in heatshocked *Tg(hs:DNfgfR1)* primordia leads to a reduced BrdU index

BrdU labeling (red) in heatshocked, nontransgenic sibs (A) and heatshocked *Tg(hs:DNfgfR1)* primordia (B), counterstained with DAPI (grey). Embryos were heatshocked at 26hpf, 6 hours prior to receiving a 1 hour BrdU pulse at 32hpf and immediately fixed. (C) Reducing Fgf signaling by heatshocking *Tg(hs:DNfgfR1)* embryos leads to a significant reduction in BrdU incorporation (BrdU index for heatshocked, nontransgenic sibs = 0.28±0.10, n=18; BrdU index for heatshocked *tg(hs:DNfgfR1)* = 0.21±0.10; n=16; Student's t-test p=0.005).

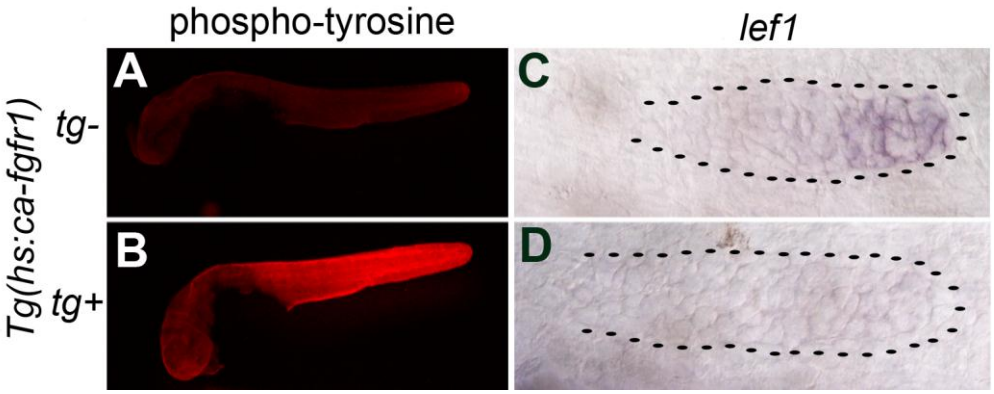


Figure 5.8 Heatshock induction of *ca-fgfr1* stimulates Fgf signaling in the primordium

(A,B) Whole mount phospho-tyrosine antibody staining at 32hpf, 6 hours post heatshock. Embryos were mounted and photographed together to accurately display intensity difference. (A) Nontransgenic sibs express phospho-tyrosine broadly. (B) Embryos harboring the *Tg(hs:ca-fgfr1)* transgene show much more intense phospho-tyrosine staining demonstrating that the transgene activates Fgf signaling throughout the animal. (C,D) In situ hybridization with the Wnt/β-catenin pathway target *lef1* in the primordium at 32hpf, 6 hours post heatshock. (C) In 32 hpf nontransgenic sibs *lef1* is expressed in the leading zone of the primordium. (D) Induction of *ca-fgfr1* leads to a loss of Wnt/β-catenin signaling in the primordium.

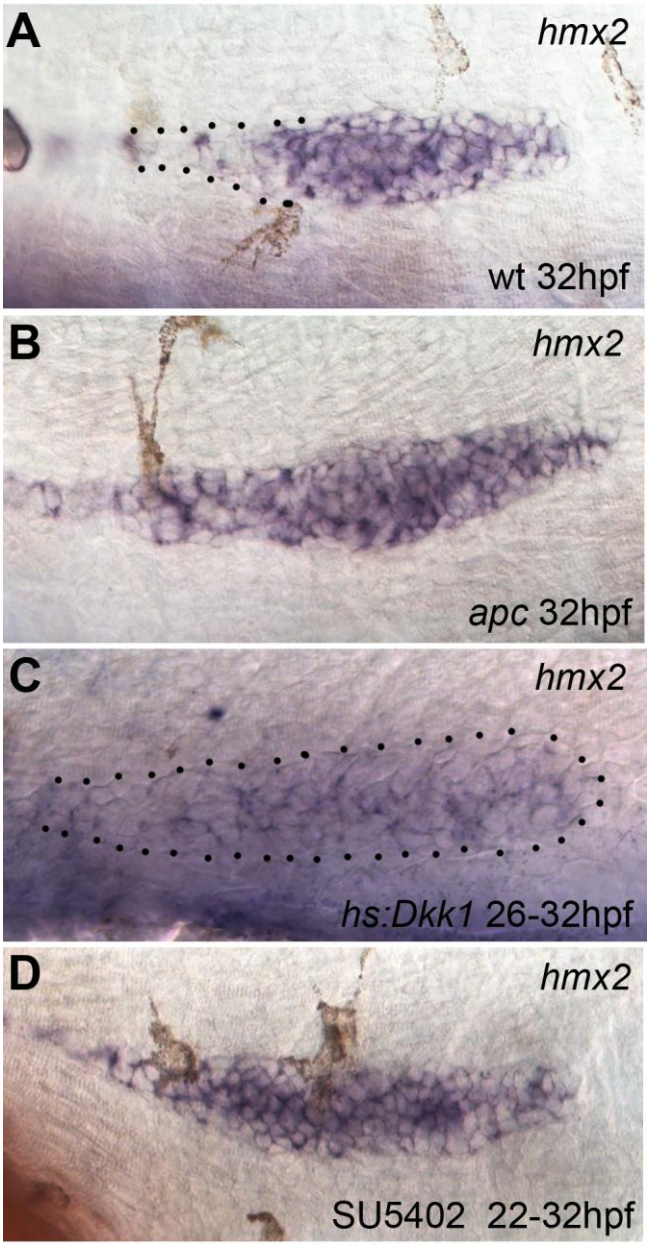


Figure 5.9 Regulation of *hmx* genes in the lateral line primordium
(A) In wt embryos *hmx2* is expressed broadly in the leading portion of the primordium but is reduced in the trailing region. (B) In *apc* mutant embryos, characterized by constitutively active Wnt/β-catenin signaling throughout the primordium, *hmx2* expression expands into the trailing region of the primordium. (C) Reducing Wnt/β-catenin signaling by heatshock induction of *dkk1* in *Tg(hs:Dkk1)* embryos leads to a downregulation of *hmx2*. (D) SU5402 treatment between 22-32hpf abolishes Fgf signaling and expands Wnt/β-catenin signaling in the primordium leading to expanded expression of *hmx2*.

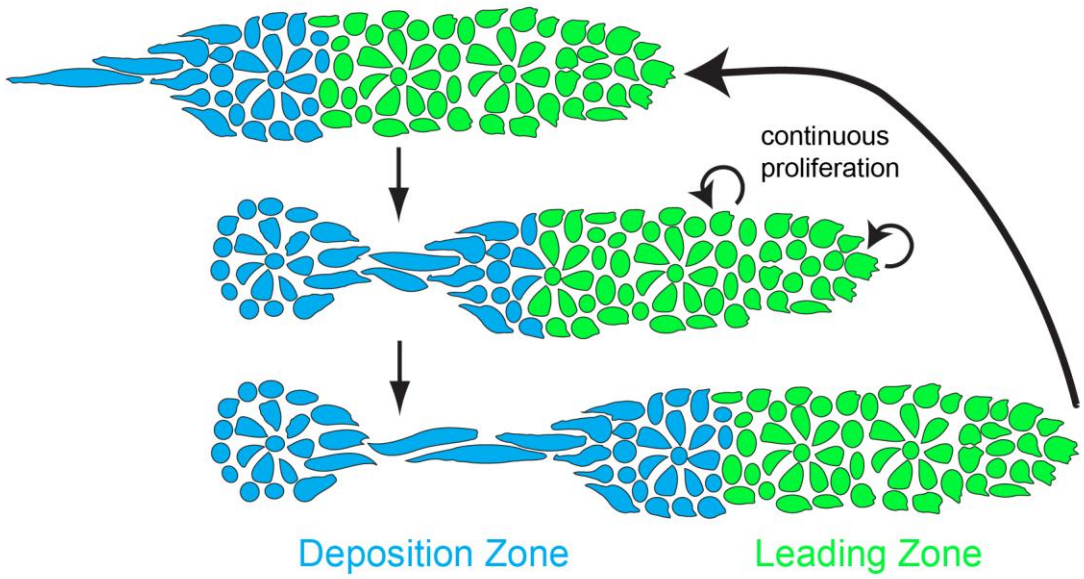


Figure 5.10. The proliferation-dependent primordium lengthening model of periodic proneuromast deposition

Proliferation in the leading zone (green) displaces cells into the deposition zone (blue) where they begin slowing down and depositing from the primordium. When an entire proneuromast is displaced into the deposition zone it deposits. This leads to a shortened primordium (middle panel). Proliferation continues, another proneuromast is displaced into the deposition zone, and the cycle repeats.

CHAPTER 6

CONCLUSIONS

Wnt/β-catenin signaling stably patterns the lateral line primordium

Data presented in Chapter 2 and discussed in Chapters 3 and 4 show the migrating primordium is stably patterned into two distinct domains. This patterning is achieved by feedback interactions between the Wnt/β-catenin and Fgf signaling pathways (Figure 2.17). In the leading region of the primordium, Wnt/β-catenin signaling is active and induces the expression of the secreted Fgf ligands *fgf3* and *fgf10* as well as the membrane bound Fgf inhibitor *sef* (Figure 2.1;2.5). *sef* expression represses Fgf signaling in leading zone cells but Fgf ligands diffuse and activate signaling via Fgfr1 in trailing cells (Figure 2.5). Fgf signaling in trailing cells induces the expression of the Wnt/β-catenin inhibitor *dkk1* thereby restricting activation of Wnt/β-catenin signaling to the leading region. Additionally, Fgf signaling in the trailing region leads to upregultion of *fgfr1,* stabilizing this domain (Figure 2.17). This feedback circuit stably patterns the primordium into distinct leading and trailing regions as it migrates and deposits proneuromasts.

Although patterning during development is often studied in terms of cell fate, the work presented in this dissertation represents a clear example of patterning contributing to morphogenesis. In this case, patterning results in the execution of distinct cell behavior programs in different regions of the primordium. In the following sections I will detail how the Wnt/β-catenin-Fgf feedback circuit coordinates cell migration, cell shape changes and cell proliferation to generate the morphology of the postembryonic lateral line.

Wnt/β-catenin signaling regulates cell migration

Distribution of numerous neuromasts along the flank of the animal is a key feature of lateral line morphology. Distribution of neuromasts is achieved by a migrating primordium that travels the entire length of the trunk, periodically depositing immature proneuromasts. The primordium migrates as a cohesive multicellular collective where all cells extend processes and migrate in a coordinated fashion.

Collective migration of the primordium requires the polarized expression of two chemokine receptors, *cxcr4b* and *cxcr7b*. *cxcr4b* is expressed broadly in the primordium and is downregulated in trailing cells and depositing tissue (David et al., 2002; Figure 2.16). Conversely, *cxcr7b* expression is restricted from the leading region and is exclusively expressed in deposited tissue and in cells that are soon to be deposited from the primordium (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Loss of either gene leads to strong defects in collective migration (Dambly-Chaudiere et al., 2007; David et al., 2002; Haas and Gilmour, 2006; Valentin et al., 2007).

Spatial restriction of Wnt/β-catenin signaling is necessary for this anisotropic expression of the chemokine guidance receptors. In the leading region, where Wnt/β-catenin signaling is active and Fgf signaling is inhibited, Wnt/β-catenin signaling represses expression of *cxcr7b*. Since Wnt/β-catenin signaling typically results in the induction of gene expression via TCF/LEF transcription factors (MacDonald et al., 2009), this repression is likely achieved by the upregulation of additional factors that inhibit transcription of *cxcr7b*. In the

trailing region, where Wnt/β-catenin signaling is repressed, *cxcr7b* is expressed and *cxcr4b* is downregulated. Manipulations that increase the extent on Wnt/β-catenin signaling into the trailing region are accompanied by failure of collective migration accompanied by loss of *cxcr7b* expression (Figures 2.1, 2.4, 2.16). These stalled primordia take on a stretched appearance that is strikingly similar to the reported *cxcr7b* morpholino phenotype, suggesting that the migration defects resulting from ectopic Wnt/β-catenin signaling may be caused by loss of *cxcr7b* expression (Dambly-Chaudiere et al., 2007; Valentin et al., 2007).

It is thought that *cxcr7b* does not transduce chemtactic signals like cxcr4b and other chemokine receptors. rather, *cxcr7b* in the trailing cells is hypothesized to serve as an Cxcl12a sink that helps to form or reinforce a gradient of Cxcl12a protein across primordium cells by binding and removing cxcl12a protein from extracellular space (Dambly-Chaudiere et al., 2007; Figure 3.1). Although this hypothesis is attractive in light of the demonstration of this mechanism during primordial germ cell migration (Boldajipour et al., 2008; Naumann et al., 2010), this model is difficult to reconcile with mosaic experiments demonstrating that only a few cells at the extreme leading edge of the primordium must respond to chemokine guidance signals trigger directional migration of all cells within the primordium (Haas and Gilmour, 2006). In this case, trailing cells that express *cxcr7b* are separated from leading cells that express *cxcr4b* by cells that that presumably express no chemokine receptors. Since *cxcl12a* appears to be uniformly expressed along the migratory trajectory, this arrangement of

chemokine receptors in mosaic primordia is unlikely to generate an informative gradient.

One way to reconcile the Cxcl12a sink hypothesis with the mosaic experiments described above is to hypothesize the existence of additional chemokine receptors in the primordium. In this scenario, all cells of the primordium continue to express some chemokine receptors, even though only a few leading edge cells express *cxcr4b*. *cxcr7b* in the trailing region depletes Cxcl12a protein from extracellular space and shapes a protein gradient that the central cells detect with the hypothetical chemokine receptor.

A second possibility is that there is mechanotactic signaling between leading edge cells and other cells of the primordium (Lecaudey and Gilmour, 2006). Such a mechanism has been demonstrated for endothelial cells migrating in response to physical stresses (Li et al., 2005). In this case, perhaps *cxcr7b* expression reduces Cxcl12a-Cxcr4b binding by competing for Cxcl12a causing trailing cells to slow and eventually stop migrating. This slowing leads to stretching forces on primordium cells aligned along the correct migratory direction and generates or reinforces mechanotactic signaling in the primordium. This mechanism could potentially operate in parallel to the Cxcl12a sink model presented above.

A third possibility suggested by (Valentin et al., 2007) is that unidentified *cxcr7b* ligands expressed in cells at the leading region trigger directional migration of trailing cells. In this scenario, when *cxcr7b* is lost trailing cells lack directional information and tumble contributing to the stretched primordial

phenotype associated with *cxcr7b* loss of function. Resolving the molecular mechanism by which *cxcr4b* and *cxcr7b* regulate collective emigration will require increased biochemical knowledge of the signaling activity, binding partners and scavenging functions of Cxcr7b as well as the elucidation of potential mechanotactic components in the lateral line.

Wnt/β-catenin signaling regulates the cell shape changes that generate proneuromasts

In order to distribute neuromasts along the trunk new neuromasts must form within the migrating primordium. Proneuromast formation requires apical constriction of cells within the primordium to generate a garlic bulb shaped cellular rosettes (Figure 1.1). At the onset of migration the primordium is composed of approximately 100 cells. The postembryonic lateral line is composed of approximately 200-300 cells (Laguerre et al., 2005). Therefore formation of proneuromasts must be balanced with maintenance of progenitor cells in order to ensure that neuromasts are distributed along the entire length of the trunk.

Data presented in Chapter 2 demonstrate that Wnt/β-catenin signaling restricts proneuromast formation to the trailing region of the primordium (Figure 2.11). Fgf signaling has been shown to promote the formation of proneuromasts by regulating components of the classical epithelial polarity machinery (Nechiporuk and Raible, 2008; Lecaudey et al., 2008; Hava et al., 2009). By shaping the region of Fgf signaling activity, Wnt/β-catenin signaling regulates the spatial distribution of rosette formation within the primordium, restricting this

behavior from cells in the leading region. This is likely to be important for maintaining a pool of proliferating progenitor cells in the leading region that can supply cells for continued proneuromast formation in the trailing region.

Wnt/β-catenin dependent cell proliferation determines the periodicity of proneuromast deposition

Another vital aspect of lateral line morphogenesis is that proneuromasts formed within the migrating primordium must be periodically deposited from its trailing edge. Periodic deposition assures that proneuromasts are uniformly distributed along the along the length of the trunk.

Spatial and temporal periodicity can be generated in the embryo by various mechanisms. In vertebrate somitogenesis cyclic gene expression underlies the segmental morphogenesis of mesoderm (Giudicelli et al., 2007). In the early drosophila embryo, gradients of diffusible morphogenesis induce a complex set of interactions of transcription factors that generate segmented patterning (Levine, 2008).

Data presented in Chapter 4 suggest a novel mechanism for generating periodicity in the embryo. In this mechanism, cell proliferation leads to continuous primordium lengthening. This causes the proneuromast at the trailing edge to be displaced beyond the reach of Wnt/β-catenin signaling. Once a proneuromast occupies this Wnt/β-catenin free deposition zone, it begins expressing *cxcr7b*, slows down and deposits from the primordium. Both cell proliferation and the

placement of the deposition zone depend on Wnt/β-catenin signaling in the primordium (Fig. 5.5, 5.6).

This model represents a novel segmentation mechanism that relies on the dynamic cellular behaviors proliferation and migration. In the primordium, these cellular behaviors are not themselves periodic. Rather, continuous migration and proliferation influence periodicity when combined with stable patterning of the primordium and regular formation of proneuromasts within the primordium.

Coordination of cell behaviors during morphogenesis

Morphogenesis involves the combined behavior of many cells to generate a genetically specified form. Mechanisms that coordinate cell behavior across space and time are therefore crucially important. Work presented in this dissertation shows that a feedback circuit involving the Wnt/β-catenin signaling pathway coordinates key cell behaviors during lateral line morphogenesis. Migration, epithelial cell shape changes and proliferation are all required for lateral line morphogenesis and all three cell behaviors are regulated by Wnt/β-catenin signaling.

This couples these behaviors during lateral during morphogenesis ensuring that they are executed as part of an orchestrated program that generates the correct morphology. Specifically, restricting Fgf signaling to the trailing region and Wnt/β-catenin signaling to the leading region drives anisotropic chemokine receptor expression and collective migration while simultaneously restricting proneuromast formation to the trailing region ensuring the maintenance of a progenitor pool at the leading edge. Likewise, the same

signaling circuit that regulates chemokine receptor expression and Fgf dependent proneuromast formation is also required for proliferation in the primordium and ultimately sets the pace of proneuromast production. This coupling ensures that the primordium will continue to generate and deposit new proneuromasts for as long as it migrates and it will only migrate as long as it can generate and deposit proneuromasts.

Disrupting either Wnt/β-catenin or Fgf signaling causes a catastrophic collapse of this signaling circuit and aberrant morphogenesis involving defects in multiple cell behaviors. This has generated some confusion in the literature where authors have presumed that proneuromast formation is required for migration (Nechiporuk and Raible, 2008; Lecaudey et al., 2008), or that proneuromast formation represses cell proliferation (Hava et al., 2009). Data presented in this dissertation demonstrate that these cell behaviors are independent but are coordinately regulated by Wnt/β-catenin signaling. Given the interconnected nature of the signaling networks that drive morphogenesis it is necessary to manipulate cell behavior directly, without impinging on cell signaling, to understand the roles on individual cell behaviors during morphogenesis.

In general, it may be expected that morphogenesis is frequently regulated by cell signaling networks that lead to elaborate patterning of tissues within developing embryos. This patterning serves to coordinate different cell behaviors across space and time ensuring the execution of a harmonious program of cellular activity and generation of the correct genetically determined

form. The lateral line represents a simple and tractable model to work out the regulatory logic of such programs. Similar regulatory logic will likely to be found in other developing structures although as the complexity of forms and the number of distinct cell behaviors increases, working out the signaling interactions and the downstream cell behaviors will represent an increasingly formidable challenge.

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